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INDEX

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INDEX

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SUPPLEMENTA

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ACUTE UNMODIFIED KIDNEY ALLOGRAFT REJECTION IN THE RABBIT

Histological and Immunofluorescent Observations

IVER HERON

Blood Grouping Laboratory and Institute of Pathology,
Municipal Hospital 9000 Aalborg Denmark

Within 1–4 days after kidney transplantation mononuclear cell infiltration developed in rabbit homotransplants The early cells aggregated around interlobular vessels and gradually infiltrated the cortex during the next few days spreading predominantly through intertubular connective tissue Swelling of endothelial cells lining medium sized vessels was noted and in transplants surviving for more than 10 days these vessels showed intimal proliferation Glomerular changes were not prominent A few immunoglobulin-containing cells could be detected by fluorescence microscopy in most of the transplants at the time of rejection The morphological changes are compared with observations made by others and differences in the results of immunofluorescence studies are pointed out It is concluded that the results of immunohistological investigations are not comparable as long as no standardization of immunofluorescence reagents and methods exists

The allograft immune reaction which leads to the destruction of whole vascularized organs is a complex patho-physiological process in part characterized by a cellular infiltrative component Mononuclear cellular proliferation in the graft has been noted to be a prominent feature of rejection (5, 8) It has been proved that cells of recipient origin constitute part of the initial cells participating in the reaction (3 18) Recently evidence has been found that the immunogenic stimulus of the host cells is of haematopoietic origin (9) probably the leucocytes in the graft (4, 7, 20)

Histological and immunohistochemical changes in kidneys have been described in dogs and inbred rats which had received renal transplants from allogeneic recipients Only few papers have dealt with observations of rejected rabbit kidneys (15, 16) It is the purpose of this report to describe the morphological changes which developed during acute unmodified kidney allograft rejection, as observed by light and fluorescence microscopy Most of the rabbits investigated in this study survived with an orthotopic kidney transplant as the only source of renal function until rejection and uraemia ended their life

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This work was supported by grants from Forskningsrådet in Denmark and from Kong Christian den Tiendes Fond

Investigations were made under a scholarship under the University of Aarhus

Requests for reprints should be addressed to I Heron Blood Grouping Laboratory Kommunehospitalet 9000 Aalborg Denmark

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MATERIAL AND METHODS

Randomly bred adult New Zealand White, Black Alaska and brown lop eared rabbits were used in the study

One group of rabbits underwent a leftsided orthotopic kidney transplantation The surgical technique has been detailed in a previous report (11) The right kidneys were removed either at the end of the transplantation or within three days after the operation

Another group of rabbits received a heterotopic kidney transplant which was placed in the neck using a technique for vascular anastomosis similar to that described for accessory heart transplantation (10) A segment of the donor s aorta was removed along with the renal artery and connected to the common carotid artery The renal vein was anastomosed to the external jugular vein A ureterocutane anastomosis with insertion of a piece of polyethylenetube in the lumen of the ureter end was made to allow urine to drain from the kidneys Abdominal nephrectomy was not carried out in this group

Male rabbits served as donors in every case and the recipients were unrelated females which had never been mated No antibiotics were used and no immunosuppressive treatment given at any time

Renal function following surgery was checked by urinary production and serum creatinine levels Recipients in the orthotopically transplanted group died in a uraemic state with cessation of urinary production within 7 to 21 days following transplantation (mean 9 6 days)

In the second group, kidney allografts were biopsied and removed at varying intervals after surgery (3 12 24, and 36 hours as well as 2 3 5 7, 8 and 10 days) to get information about the chronological sequence of the morphological changes in individual kidneys

On removal the kidneys were examined macroscopically and representative tissue was taken for light and fluorescencemicroscopy For the former tissue was placed in formalin 10 per cent for the latter, tissue was quick frozen and stored at —24° C

More than 35 renal transplants were available for serial study from the day of transplantation to 21 days after

Tissue controls consisted of 1) orthotopically autotransplanted kidneys 2) kidneys removed at the time of transplantation 3) the recipient s second kidney removed 1 3 days post operatively and 4) biopsies from the right kidneys of the donors

The formalin fixed tissues were cut in an ordinary microtome and stained with hæmatoxylin eosin van Gieson periodic acid, Schiff and methyl green pyronine

The frozen tissues were sectioned in a cryostat at approximately minus 25° C Sections were air dried for 10 minutes and washed in phosphate buffered saline (pH 7 1) for 10 minutes

FITC conjugated horse antirabbit immunoglobulins and swine antirabbit immunoglobulins were commercially produced reagents* By immunoelectrophoresis the former gave two precipitin arcs, one in the IgG region the other in the IgA region Two precipitin arcs from the swine antirabbit immunoglobulin reagent were obtained one in the IgG and one in the IgM region

Sections were incubated with a few drops of labelled conjugate diluted 1 10 and left for 30 minutes in a moist chamber at 20° C After washing in buffered saline (pH 7 1) for one hour, a coverglass was mounted over a drop of buffered glycerol Control sections consisted of 1) sections previously incubated with antirabbit gammaglobulins not bound to FITC, followed by washing and staining with labelled antibodies 2) sections previously incubated with non immune serum from swine followed by washing and staining Fluorescence considered specific was considerably diminished in 1) and unaltered in 2) If the fluorescence was diminished following 2) it was registered as non specific immunologically A third control section was incubated with labelled antibodies treated with the antigens *i e* rabbit immunoglobulins

The sections were studied in a Leitz Ortoplan microscope with an HBO 200 bulb as a light source Darkfield condenser was employed and photographs were taken using an Ilford FP4 film

RESULTS

Among 30 recipients in the orthotopically transplanted bilateral nephrectomy group, 10 animals were allowed to live until they underwent a spontaneous uraemic death due to rejection The length of survival differed considerably *i e* from 7 to 20 days, with an average of 9 6 days In most instances the kidneys continued to produce urine until 1–2 days before death

Kidneys in the accessory transplanted group were found to continue to produce urine for 5 8 days

The removed kidneys were enlarged swollen pale and oedematous at the time of cessation of urinary production The weight

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* Centraal Laboratorium van de Bloedtransfusiedienst van Het Nederlandsche Roede Kruis and Nordic Pharmaceuticals and Diagnostics

of the kidneys was found to be increased up to 2 to 3 times the original weight

Light Microscopy

The most prominent morphological change found in the homotransplants was infiltration with mononuclear cells

There was a considerable variation in the number of infiltrating cells present in different renal allografts at any given time after transplantation It was obvious that infiltrations in kidneys from rabbits in the nephrectomized group never appeared as early as in the accessory transplanted group and were not either as marked at the time of rejection

The biopsy specimens from individual kidneys in the latter group showed that the infiltrative changes were steadily progressive The degree of variation within kidneys in each group was most marked in early biopsies, whereas later specimens showed slight variation only

The earliest mononuclear cell infiltration of the homograft was observed 24 hours after surgery Most cases revealed infiltration within 2 3 days and in one case no infiltrative component was seen at day 4 after transplantation

In the perivascular areas of the connective tissue the cells accumulated round interlobular renal vessels These cells had the morphological appearance of small and medium sized lymphocytes and mononuclear cells with vesicular nuclei and prominent nucleoli A small number of the latter exhibited pyroninophilic cytoplasm The accumulation of lymphoid cells progressed in number during the next days (Fig 1) The cortex was progressively infiltrated cells appearing first around smaller vessels and between the tubules Six days after transplantation the renal cortex in the quickest cases might be diffusely infiltrated with cells (Fig 2) Increasing numbers of mature plasma cells could be detected in the aggregates of mononuclear cells from day 5 on The predominant cell type found at any time in the inflammatory infiltrate appeared to be non pyroninophilic mononuclear cells measuring 10-15 m diameter with large irregular vesicular nuclei Eosinophilic granulocytes and polymorphonuclear leucocytes were occasionally seen the latter increasing in number in the late stages At the time of anuria the kidneys showed evidence of early infarction with interstitial oedema and occasional foci of interstitial haemorrhage

Tubular epithelial cells showed increasing intensity of degenerative changes including coarse vacuoles swelling of the cytoplasma, and loss of brush border Schiff positive tubular casts were frequently seen Usually some dilatation of the distal tubules occurred, the cells showing droplet degeneration vacuolation and necrosis

In the terminal stages vascular changes were found consisting mostly of swelling of the endothelial cells lining the smaller arteries

Proliferation of endothelial cells lining the interlobular arteries was found in a few cases and was most prominent in kidneys rejected late The kidney that continued to produce urine for 18 days showed monstrous endothelial proliferation within all interlobular arteries seen in the sections (Fig 3) A few arteries were seen to be totally occluded by these proliferations

Fibrinoid necrosis and medial vacuolization was never found with certainty in any vessel wall Possible destruction or disruption in patency of intertubular capillaries could not be evaluated in the light microscope because of the heavy cellular infiltration in these areas

Most glomeruli did not undergo changes Occasionally slight hypercellularity and some increase in Schiff positive mesangial matrix was noted In rare cases proliferating cells caused a development of partly avascular glomeruli (Fig 4)

Control renal autografts removed after 7 and 10 days revealed only minimal changes, consisting in scattering of single mononuclear cells in the cortical connective tissue septa and the presence of a few tubular casts

Host kidneys removed at the time of transplantation served as a baseline control for

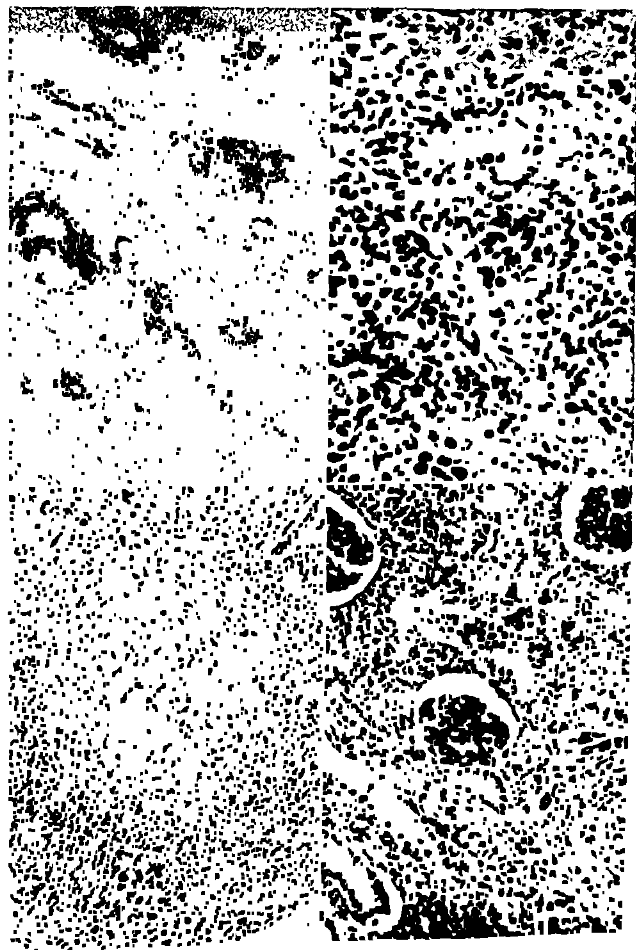


Fig 1 Cortical mononuclear cell infiltration in a homotransplant on day 5 Note heavy infiltrates around the greater vessels (haematoxylin and eosin × 40)

Fig 2 Diffuse cellular infiltration in an allograft on day 7 Note mature plasmacells with pyroninophilic cytoplasm (methyl green pyronine × 400)

Fig 3 Artery showing pronounced intimal proliferation in a renal transplant which functioned for 18 days (haematoxylin and eosin × 250)

Fig 4 Section from a kidney allograft 7 days after transplantation A slightly hypercellular glomerulus is seen capillary loops are occluded and mesangium contains increased Schiff positive material (Periodic acid Schiff × 250)

morphological alterations It was striking that the kidneys in a few rabbits otherwise considered healthy revealed slight glomerular hypercellularity In two host kidneys removed after rejection similar changes were found

Immunofluorescence Microscopy

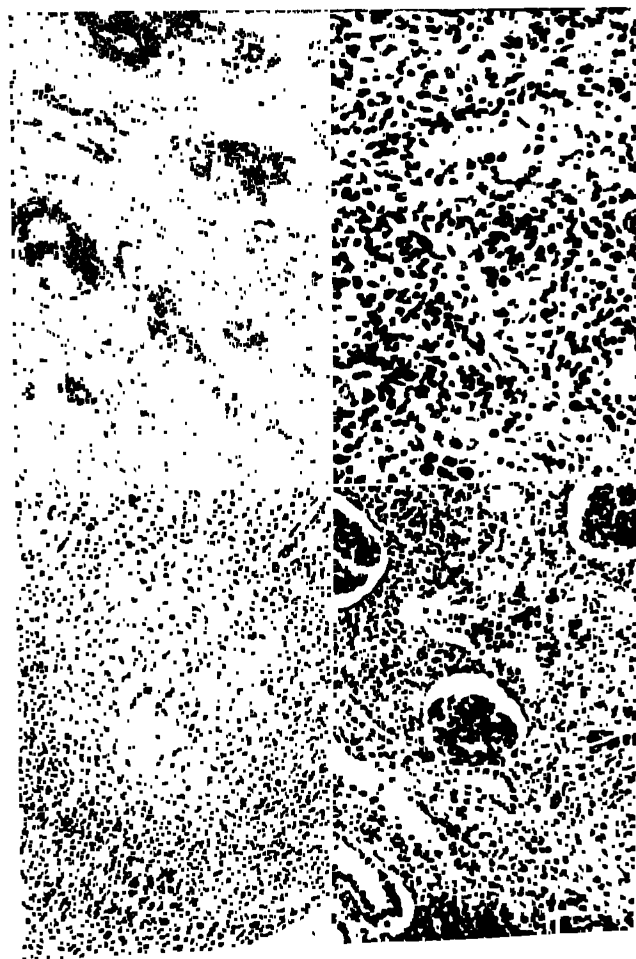
If specific fluorescence was obtained to the same extent after staining with the anti sera against IgG IgA and IgG IgM, it was taken as evidence that γ globulins were present If staining occurred with only one of the reagents it was considered that IgG was absent Absence of staining for IgG-IgA, but positive staining for IgG IgM was considered to signify the presence of IgM globulin

No changes were found to be constantly related to and pathognomonic for allograft rejection Tubular casts exhibiting fluorescence for γ globulins could be found in every allograft but might also occur frequently in autografted kidneys Interstitial and inter- and peritubular γ globulin reactions were found in allografts as well as in autografts and normal control kidneys the morphological appearance being somewhat different owing to a change in architecture of the homografts γ globulins could be traced to the walls of medium sized vessels in allografts only in very few instances In these cases fluorescence was seen in a diffuse pattern throughout all layers

Glomerular fluorescence for γ globulins showed two different patterns in the kidneys

TABLE 1 *Summary of Histological Findings in the Homograft*

	Cellular infiltrate	Tissue necrosis/ infarction	Degenerative tubular changes	Vascular changes	Glomerular changes
Days 1–4	Perivascular lymphocytes	—	+		—
Days 4–6	Large mononuclear and plasmacells	+	+ +	Swelling of endothelial cells and oedema	—
Days 6–12	Diffuse interstitial obliteration Appearance of macrophages and granulocytes	+ + +	+ + +	Ditto	Occasional hypercellularity and increase in mesangial matrix
Days 12 20	Ditto	+ + +	+ + +	Endothelial cell proliferation and vascular obliteration	Ditto



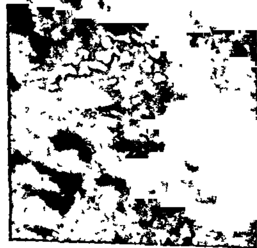


Fig 7 Cluster of mononuclear cells in allotransplant on day 4 Cytoplasmatic fluorescence is present following staining with labelled antibodies against IgG IgM No fluorescence of these cells was seen when stained for IgG IgA (× 400)

for this is obscure but it might be explained by chance genetic compatibility between host and donor Similar unexpectedly prolonged functional survival of kidneys in dogs receiving homologous renal transplants has been reported (13 21)

The histological changes as observed in the light microscope have been compared with some of those observed in canine and rat allografting experiments on unmodified recipients previously reported (2 5 14, 19) On the basis of these findings the morphological changes in renal transplants seem to differ only slightly between species and are mostly related to time

The observation made in the present study that cellular infiltration in the graft appeared later and became less marked in nephrectomized hosts as compared with hosts with adequate and normal renal function during rejection of accessory transplants is interesting, as function of the transplant i e the ability to secrete urine, ordinarily ceased within the same length of time in both groups These observations indicate that mononuclear cell infiltration in the graft is dependent to a certain extent on the kidney function of the host whereas the fundamental processes involved in rejection differ to some extent and are either non-dependent on kidney function or take place and govern the process of rejection at a time when kidney function is similar in the two groups i e within the first few days following transplantation

As regards immunohistochemical observations the results published so far differ considerably *Horowitz et al* (12) have studied homotransplanted dog kidneys and found γ globulins in the walls of smaller vessels as well as complement binding ability They found few γ globulin containing cells of immature plasma cell type on days 2–3, and numerous predominantly mature, plasma cells containing cytoplasmatic γ globulin on days 4–5 *Fisher & Fisher* (6) were unable to demonstrate specific fluorescence in dog kidneys undergoing rejection using a fluorescent labelled anti dog globulin as immunological reagent *Feldman & Lee* (5) investigated homologous renal transplants in Lewis rats and only rarely found cells in the infiltrate stained by either anti IgG or anti IgM globulins Mesangial staining was frequently found but was considered to be of no immunological significance *Lindquist et al* (14) made immunohistochemical studies of acute allograft rejection in rats utilizing a strain combination giving pure host versus graft reaction These workers observed IgG containing mononuclear cells within peritubular capillaries but reported an absence of γ-globulin containing cells in the mononuclear cell infiltrates during the first week within which urinary production ceased 2–3 days after transplantation IgG was however



Fig 5 Glomerulus in a kidney from a normal control rabbit stained with FITC conjugated horse antirabbit immunoglobulin Note linear deposits along basement membrane (× 250)

investigated One was linear smooth deposits along the basement membranes (Fig 5) This pattern was seen in a few of the normal donor control kidneys and was found also in two allotransplants throughout the first 3 days following transplantation but could not be seen later The second pattern of glomerular γ globulin fluorescence appeared as granular mesangial deposits (Fig 6) present in approximately 10 per cent of the glomeruli in half the allotransplants This fluorescence was most prominent on days 4 6 and usually disappeared when kidney function ceased totally

Mononuclear cells exhibiting fluorescence for γ globulins were found only in two biopsies of allografts after three and four days respectively In the one case the cells were single resembling typical lymphocytes in the other, the cells were richer in cytoplasm and they appeared in small clusters some of which were localized intravascularly Mononuclear cells exhibiting cytoplasmatic staining considered to be IgM specific could be found in increasing numbers in later stages of the homotransplants (Fig 7) They were never nearly as numerous as the cells with pyroninophilic cytoplasm These cells could be detected in 90 per cent of rejected kidneys and were found to be of two morphological types The one type occured rarely, and was found scattered as single cells with the morphology of typical plasma cells The other type was seen more frequently and appeared in clusters perivascularly, looking like histiocytes or macrophages

DISCUSSION

In the present study the homotransplanted kidneys were found to function for periods that did not differ much from those reported by others (15 16 17) The case in which a Black Alaska recipient of a kidney from a New Zealand white was seen to survive for 21 days was rather unexpected The reason



Fig 6 Glomerulus in a renal homotransplant on day 6 γ globulins are present in a granular pattern predominantly within mesangium (FITC conjugated swine antirabbit immunoglobulin × 400)



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located on the walls of peritubular capillaries and vascular walls as was also the βIC globulin

Renal allografts in the immunosuppressed human subject show a somewhat different pattern of staining but this cannot be considered comparable to allografting experiments in unmodified animals

In general attention should be direct to the difficulties involved when immunofluorescence staining reactions are to be considered of immunological significance especially in cryostat sections with numerous artefacts Immunological reagents used in separate studies are different and no standardization of these exists As long as reagents washing procedures and controls differ it is my opinion that results are in fact not comparable In the present study it was found that peritubular capillaries during the allograft rejection phase contained γ globulins Careful investigations of tissue controls and autotransplants and also experience with intensified washing prior to and following staining with labelled antibodies *in different dilutions* made the author conclude that the fluorescence of the capillaries was not of immunological significance

Mononuclear cells exhibiting positive fluorescence for IgG globulins were numerous in fresh cryostat sections using one commercially produced FITC labelled antirabbit antibody The commonly used blocking control procedure involving exposure of sections to unlabelled antibodies prior to exposure to the labelled ones was *used and supported the* assumption that the fluorescence was specific When however an extra blocking control procedure was carried out with a non immune serum from the same species prior to incubation with labelled immune serum fluorescence of a great amount of *the* mononuclear cells was again considerably reduced Therefore fluorescent staining could not be considered specific The explanation of these observations must be that some cells in fresh *frozen sections* absorb proteins for non immunological reasons These examples clearly demonstrate the consequences of even small differences in control procedures to the final interpretation of the results

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The valuable help by *A Richter Nielsen* in interpreting the histological material is gratefully acknowledged

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Acta path microbiol scand Section A 79, 10–14, 1971

PRIMARY HYPERPARATHYROIDISM IN A CASE OF OXYPHILIC ADENOMA

Clinical and Morphological Observations

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A 47 year old man with clinical manifestations of hyperparathyroidism was shown to have a parathyroid adenoma which in light microscopy chiefly consists of oxyphilic cells and transitional forms of chief cells and oxyphilic cells Electron microscopy shows that the oxyphilic cells contain tightly packed mitochondria and that the transitory cells are rich in mitochondria In these latter cells there are signs of stimulated protein synthesis It is probable that these cells produce the excess of parathyroid hormone Oxyphilic cells would seem to be developed from the chief cells

Oxyphilic cells have been considered to be inactive during involution In the literature however, there are reports of hyperfunctioning oxyphilic adenomata (12) Certain authors (2, 3) consider that the hyperfunctioning oxyphilic adenoma has to be interpreted as a mixed tumour *Selzman & Fechner* (1967) definitely maintain that the cells rich in mitochondria, in their case of oxyphilic adenoma, have high hormone producing activity Against the background of the discussion around clinical and theoretical importance of the oxyphilic adenomata we describe a case of clinically manifest hyperparathyroidism with a predominant oxyphilic parathyroid adenoma

CASE DESCRIPTION

The patient is a 47 year old man who consulted a doctor because of retrosternal aginal pains Ten years ago the patient had suffered from four attacks of renal colic During later years the patient has suffered from increased irritability feelings of discomfort depressed state of mind and tiredness

On investigation coronary insufficiency was found Serum calcium varied between 6 2 and 6 5 mE/l serum phosphorus between 1 0 and 1 2 mE/l and calcium excretion in the urine between 237 and 364 mg/day Slightly raised serum alkaline phosphatase normal electrophoresis normal serum magnesium Electrolyte state otherwise normal Normal X ray of skeleton and lungs No goitre The patient was euthyroid Urography showed a great number of calculi size ranging from that of a half pea to grain size in both kidneys characteristic of nephrocalcinosis

By exploration of the neck a red brown parathyroid tumour measuring 12 × 9 × 5 mm was found on the left side between the lower thyroid pole and the recurrent nerve A large branch from the lower main stem of the inferior thyroid artery supplied the parathyroid tumour All the other parathyroid glands were identified and were macroscopically normal One of these normal parathyroid glands was removed for histo pathological investigation

The post operative course was free from com

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plications with prompt normalization of the serum calcium

Light and Electron Microscopical Studies

Both preparations were embedded in paraffin and cut at several levels in 3 μ thick sections The sections were stained according to van Gieson and with haematoxylin-eosin

At the operation a little piece of the parathyroid adenoma as well as half the normal parathyroid gland was taken for electronmicroscopic investigation After prefixation in 3 per cent glutaraldehyde in 0 075 M Cacaodylate buffer at pH 7 2 for two hours definite fixation took place in 1 per cent OsO_4 in 0 028 M veronal acetate pH 7 15 during a similar period of 2 hours After dehydration the specimens were embedded in Epon 812 sectioned with glass knives in a LKB Ultrotome and stained with uranyl and lead acetate Electron microscopy was carried out in a Siemens Elmiskop I with double condenser and an accelerating voltage of 80 kV

Histologically a parathyroid adenoma with a rich vascularity was observed The adenoma was built up chiefly of oxyphilic cells and transitional forms of chief and oxyphilic cells Both these cell forms had an eosinophilic cytoplasm strongest in ... In a number of areas the cells

cell groups were surrounded by an oedematous stroma deficient in cells In the edge of the adenoma close to the artery, the rest of the original parathyroid gland consisting of chief cells was observed

The normal parathyroid gland was fat infiltrated and built up in its entirety of light chief cells of uniform type The cells formed alveolar or in some cases trabecular structures

In the chief cells of the normal parathyroid gland the nucleus occupied the greater part of the cell volume The endoplasmic reticulum was sparsely developed but free ribosomes occurred in relatively large numbers The few mitochondria were relatively small and showed less form variation

In the oxyphilic cells of the adenoma the cytoplasm was nearly completely occupied by large irregularly formed mitochondria with closely spaced inner membranes Gly...

served in the cytopla... chondria (Fig 1) Th... trally situated and su... clear membrane In s... extremely large number of the endoplasmic r... longing to the Golgi a...

The ultrastructure of the transitional forms of chief and oxyphilic cells could be summarized as chief cells with clusters of mitochondria scattered in the cytoplasm The endoplasmic reticulum was well developed with narrow, longish cisternae and a large number of free as well as membranebound ribosomes The Golgi apparatus was wide spread and some of the saccules were large In the periphery of the Golgi apparatus a large number of small vesicles were observed (Fig 2) The mitochondria were of varying size and form and their matrix was of relatively high electronoptic density Fundamentally there was no difference between the ultrastructure found in these transitional cells and that found in the mitochondrion rich cells of the adenoma

DISCUSSION

The majority of cases of hyperparathyroidism are caused by adenomata, but among these, those consisting mainly of oxyphilic cells are rare Thus, in our study of more than 250 cases of hyperparathyroidism (10) we have only observed this single case, which was supplied by an unusually large artery and had a pronounced red brown colour as an expression of its rich vascularity

Oxyphilic cells occur essentially in thyroid, parathyroid and salivary glands The ultrastructure of oxyphilic cells in the human thyroid has been described by Heimann (1966 1969) The function of these cells is, in spite of the fact that they have been known since the end of the 19th century (1) as yet unknown An abundance of oxidative enzymes has been shown in oxyphilic cells in the parathyroid (13) and agrees well with the observation that these cells contain tightly packed irregularly formed large, and dense mitochondria (11) The large number of mitochondria indicates a high kinetic activity The sparse occurrence of endoplasmic reticulum and Golgi apparatus makes it probable that these cells have no great protein synthesis In the thyroid these cells do ... up radio iodine (8) It has there... n surprising that in some cases one ... able to find oxyphilic adenoma with ... ced hyperparathyroidism (12) ... itional forms of chief cells and oxy... ells have been described by ... et al

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A 47 year old man with clinical manifestations of hyperparathyroidism was shown to have a parathyroid adenoma which in light microscopy chiefly consists of oxyphilic cells and transitional forms of chief cells and oxyphilic cells. Electron microscopy shows that the oxyphilic cells contain tightly packed mitochondria and that the transitory cells are rich in mitochondria. In these latter cells there are signs of stimulated protein synthesis. It is probable that these cells produce the excess of parathyroid hormone. Oxyphilic cells would seem to be developed from the chief cells.

Oxyphilic cells have been considered to be inactive during involution. In the literature, however, there are reports of hyperfunctioning oxyphilic adenomata (12). Certain authors (2, 3) consider that the hyperfunctioning oxyphilic adenoma has to be interpreted as a mixed tumour. *Selzman & Fechner* (1967) definitely maintain that the cells rich in mitochondria in their case of oxyphilic adenoma have high hormone producing activity. Against the background of the discussion around clinical and theoretical importance of the oxyphilic adenomata we describe a case of clinically manifest hyperparathyroidism with a predominant oxyphilic parathyroid adenoma.

CASE DESCRIPTION

The patient is a 47 year old man who consulted a doctor because of retrosternal anginal pains. Ten years ago the patient had suffered from four attacks of renal colic. During later years the patient has suffered from increased irritability, feelings of discomfort, depressed state of mind and tiredness.

On investigation coronary insufficiency was found. Serum calcium varied between 6.2 and 6.5 mE/l, serum phosphorus between 1.0 and 1.2 mE/l and calcium excretion in the urine between 237 and 364 mg/day. Slightly raised serum alkaline phosphatase, normal electrophoresis, normal serum magnesium. Electrolyte state otherwise normal. Normal X-ray of skeleton and lungs. No goitre. The patient was euthyroid. Urography showed a great number of calculi, size ranging from that of a half pea to grain size, in both kidneys, characteristic of nephrocalcinosis.

By exploration of the neck a red-brown parathyroid tumour measuring 12 × 9 × 5 mm was found on the left side between the lower thyroid pole and the recurrent nerve. A large branch from the lower main stem of the inferior thyroid artery supplied the parathyroid tumour. All the other parathyroid gland were identified and were macroscopically normal. One of these normal parathyroid glands was removed for histo-pathological investigation.

The post-operative course was free from com-

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plications with prompt normalization of the serum calcium

Light and Electron Microscopical Studies

Both preparations were embedded in paraffin and cut at several levels in 3 μ thick sections The sections were stained according to van Gieson and with haematoxylin eosin

At the operation a little piece of the parathyroid adenoma as well as half the normal parathyroid gland was taken for electronmiscroscopic investigation After prefixation in 3 per cent glutaraldehyde in 0 075 M Cacadylate buffer at pH 7 2 for two hours definite fixation took place in 1 per cent OsO_4 in 0 028 M veronal acetate pH 7 15 during a similar period of 2 hours After dehydration the specimens were embedded in Epon 812 sectioned with glass knives in a LKB Ultrotome and stained with uranyl and lead acetate Electron microscopy was carried out in a Siemens Elmiskop I with double condenser and an accelerating voltage of 80 kV

Histologically a parathyroid adenoma with a rich vascularity was observed The adenoma was bo l m h aft c

showed a slight pleomorphism and among the oxyphilic cells appeared also solitary chief cells with light cytoplasm Here and there the cells formed alveolar or follicular structures but on the whole cell groups were surrounded by an oedematous stroma deficient in cells In the edge of the adenoma close to the artery the rest of the original parathyroid gland consisting of chief cells was observed

The normal parathyroid gland was fat infiltrated and built up in its entirety of light chief cells of uniform type The cells formed alveolar or in some cases trabecular structures

In the chief cells of the normal parathyroid gland the nucleus occupied the greater part of the cell volume The endoplasmic reticulum was sparsely developed but free ribosomes occurred in relatively large numbers The few mitochondria were relatively small and showed less form variation

In the oxyphilic cells of the adenoma the cytoplasm was nearly completely occupied by large irregularly formed mitochondria with closely spaced inner membranes Glycogen granules were observed in the cytoplasm between these mitochondria (Fig 1) The nucleus was large centrally situated and surrounded by a double nuclear membrane In sections from cells with an extremely large number of mitochondria cisternae of the endoplasmic reticulum or structures belonging to the Golgi-zone could not be seen

The ultrastructure of the transitional forms of chief and oxyphilic cells could be summarized as chief cells with clusters of mitochondria scattered in the cytoplasm The endoplasmic reticulum was well developed with narrow longish cisternae and a large number of free as well as membranebound ribosomes The Golgi apparatus was wide spread and some of the saccules were large In the periphery of the Golgi apparatus a large number of small vesicles were observed (Fig 2) The mitochondria were of varying size and form and their matrix was of relatively high electronoptic density Fundamentally there was no difference between the ultrastructure found in these transitional cells and that found in the mitochondrion rich cells of the adenoma

DISCUSSION

The majority of cases of hyperparathyroidism are caused by adenomata but among these, those consisting mainly of oxyphilic cells are rare Thus in our study of more than 250 cases of hyperparathyroidism (10) we have only observed this single case which was supplied by an unusually large artery and had a pronounced red brown colour as an expression of its rich vascularity

Oxyphilic cells occur essentially in thyroid parathyroid and salivary glands The ultrastructure of oxyphilic cells in the human thyroid has been described by *Heimann* (1966 1969) The function of these cells is in spite of the fact that they have been known since the end of the 19th century (1) as yet unknown An abundance of oxidative enzymes has been shown in oxyphilic cells in the parathyroid (13) and agrees well with the observation that these cells contain tightly packed irregularly formed large, and dense mitochondria (11) The large number of mitochondria indicates a high kinetic activity The sparse occurrence of endoplasmic reticulum and Golgi apparatus makes it probable that these cells have no great protein synthesis In the thyroid these cells do not take up radio iodine (8) It has therefore been surprising that in some cases one has been able to find oxyphilic adenoma with pronounced hyperparathyroidism (12)

Transitional forms of chief cells and oxyphilic cells have been described by int al

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PRIMARY HYPERPARATHYROIDISM IN A CASE OF OXYPHILIC ADENOMA

Clinical and Morphological Observations

Peter Heimann, Goran Hansson and Olov Nilsson

The Department of Surgery II, Pathology II and Anatomy, University of Goteborg Sweden

A 47 year-old man with clinical manifestations of hyperparathyroidism was shown to have a parathyroid adenoma which in light microscopy chiefly consists of oxyphilic cells and transitional forms of chief cells and oxyphilic cells Electron microscopy shows that the oxyphilic cells contain tightly packed mitochondria and that the transitory cells are rich in mitochondria In these latter cells there are signs of stimulated protein synthesis It is probable that these cells produce the excess of parathyroid hormone Oxyphilic cells would seem to be developed from the chief cells

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Histologically a parathyroid adenoma with a rich vascularity was observed The adenoma was built up chiefly of oxyphilic cells and transitional forms of chief and oxyphilic cells Both these cell forms had an eosinophilic cytoplasm *strongest in* the oxyphilic cells In a number of areas the cells showed a slight pleomorphism and among the oxy philic cells appeared also solitary chief cells with *light cytoplasm* Here and there the cells formed alveolar or follicular structures but *on the whole* cell groups were surrounded by an oedematous stroma deficient in cells *In the edge of the* adenoma close to the artery the rest of the ori ginal parathyroid gland consisting of chief cells was observed

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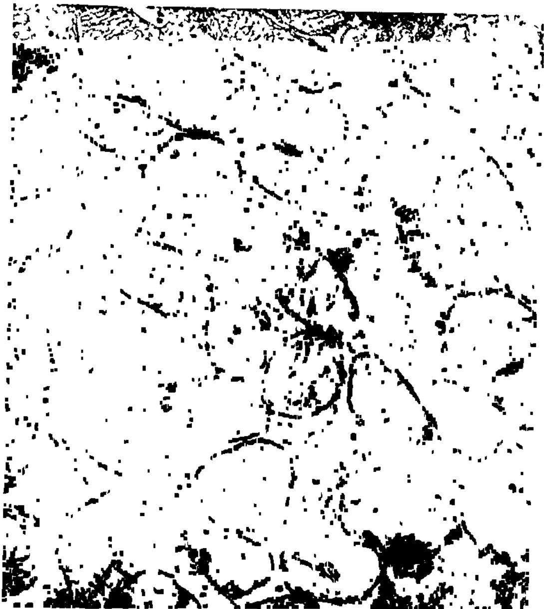


Fig 1 A mitochondrion-rich cell from the oxyphilic parathyroid adenoma Notice the rich variation in size and form of the mitochondria and the closely spaced inner membranes Between the mitochondria glycogen granules can be observed (× 43 000)

Engfeldt et al (1959), *Munger* & *Roth* (1963)

Christie (1967) contradicted the idea prevalent in the earlier literature, that oxyphilic cells are a degenerating form of chief cells As a matter of fact the oxyphilic cells seem to be very active cells The following facts support this theory

1) Oxyphilic adenomata are always rich in vessels in conformity with the case which we have described here

2) Mitochondrion-rich cells which only in exceptional cases show signs of degeneration

3) In oxyphilic cells one can demonstrate strong histochemical reactions for phospholipids, comparable with those in the acidophilic cells of the pituitary gland (4)

4) The occurrence of oxidative enzymes (13) in oxyphilic cells speaks in favour of activity

This work was supported by the *Swedish Cancer Research Council*



Fig 2 Oxyphilic parathyroid adenoma This picture shows a transitory form between chief cell and oxyphilic cell If the cytoplasm are heaps of mitochondria, which vary in size and form To the left the regularly arranged, well-developed endoplasmic reticulum is observed Between the endoplasmic reticulum and the nucleus a large Golgi zone with saccules of varying sizes and a large number of small vesicles can be observed (× 15 000)

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CORRELATION BETWEEN INFILTRATION OF MONONUCLEAR CELLS AND PRODUCTION OF CONNECTIVE TISSUE IN ACUTE HYPERTENSIVE VASCULAR DISEASE

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Damaged mesenteric arterioles from rats made hypertensive with intravenous injections of angiotensin showed a production of Van Gieson positive connective tissue which was proportional to the infiltration of mononuclear cells (lymphocytes and monocytes) Rats treated with antithymocyte serum before hypertensive damage of the arterioles showed no or only a very sparse cellular reaction and corresponding to this decreased cellular reaction no production of connective tissue fibrils was observed As some fibroblast like cells contained carbon particles in their cytoplasm it is highly probable that they were transformed lymphocytes and/or monocytes which had phagocytized carbon particles from the hypertensively damaged arterioles

Animals with experimental renal hypertension show damage of arterioles characterized by fibrinoid necrosis and periarteriolar cellular infiltration (*Schloss* 1948, *Allison et al* 1967 *Kojimahara* 1967) The cellular infiltration was interpreted as an important factor in the healing of the arterioles The fibrinoid necrosis is a reversible condition, disappearing a variable time after the blood pressure has returned to a normal level After disappearance of the fibrinoid the arterioles show either no or in a variable degree a cellulofibrous intimal and/or medial, thickening (*Allison et al* 1967, *Kojimahara* 1967)

Acute hypertensive damage of mesenteric arterioles in rats is characterized by an infiltration of plasma proteins into the arteriolar walls and followed by a cellular reaction composed of polymorphous leucocytes and mononuclear cells derived from the blood The mononuclear cells seem to be phagocytic (*Olsen* 1970a and b) It was further demonstrated that a cellular infiltration like that seen in chronic hypertension could be found in some arterioles when the duration of the hypertensive period was prolonged by repeating it throughout four consecutive days In these arterioles some of the infiltrating cells showed a morphology like fibroblasts and

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some production of connective tissue apparently had taken place. Therefore, the aim of the present work has been to study 1) the production of the connective tissue in hypertensively damaged arterioles, 2) whether such a production can be correlated to the infiltration of the mononuclear cells and 3) whether the mononuclear cells show morphological transformation to fibroblast-like cells.

MATERIAL AND METHODS

Animals. White female rats weighing 180–200 grams.

Anaesthesia. 25 mg of pentymal sodium intraperitoneally. A larger dose was used when the rats were killed.

Colloidal carbon particles. Specially produced for experimental use. (Gunther Wagner Pelikan Werke, Hannover, Germany C 11/1431 a).

Angiotensin II amid. Hypertensin CIBA dissolved in physiological saline.

Antithymocyteserum. Produced as rabbit anti rat thymocyte serum.

The experimental technique. Group 1. The small intestine of three *normal* rats was fixed in buffered formalin. Ten mesenteric arterioles from each rat were isolated, embedded in paraffin, cut in 5 micron thick serial sections and stained for connective tissue using the method of Van Gieson Hansen. From each arteriole about 20 sections were studied.

Group 2. After complete anaesthesia had been induced, a catheter of polyethylene was placed in both femoral veins in three rats. Through the one catheter *angiotensin* was injected intravenously in a dose of ½ 1 microgram *every fifth minute during 5 hours*. At the end of the injections 0.1 ml of colloidal carbon particles was injected intravenously through the other catheter. The rats were killed 24, 24 and 48 hours after the termination of the hypertensive period. The small intestines were fixed, about ten mesenteric arterioles with carbon particles deposited in the wall were isolated from each rat, embedded, cut and stained as described under group 1.

Group 3. Three rats were treated with intravenous injections of angiotensin as described under group 2, but eight days later a second treatment with angiotensin during five hours was given. At the end of this second treatment colloidal carbon particles were injected intravenously. This procedure was used because the second cellular infiltration is more marked than the primary infiltration (*Olsen* 1970c). The rats were killed 24, 48 and 48 hours after the termination of the second hypertensive period. The small intestines were fixed, about ten mesenteric arterioles with carbon particles were isolated from each rat, embedded, cut and stained as described under group 1.

Group 4. Following intravenous injections of angiotensin as described under group 2 angiotensin was injected again 10 days later in five rats during three, four and five consecutive days. These rats were killed either immediately after such treatment or 24 or 48 hours later. In the middle of the periods of the secondary injections of angiotensin, colloidal carbon particles were injected intravenously. The small intestines were fixed, about ten mesenteric arterioles with carbon deposits were isolated from each rat, embedded, cut and stained as described under group 1.

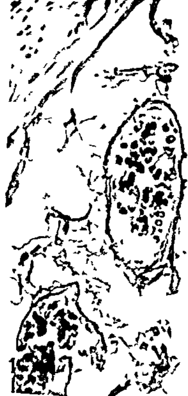
Group 5. Two rats were treated with injections of angiotensin as described under group 3, but 24 hours before and immediately before the second treatment with angiotensin, one ml of antithymocyteserum was injected intravenously. The rats were killed 48 hours after the termination of the second treatment and the small intestines were fixed in buffered formalin. Eleven mesenteric arterioles from the two rats with carbon deposits were isolated, embedded, cut and stained as described under group 1.

Fig. 1. The natural existence of Van Gieson positive connective tissue fibrils surrounding a normal arteriole situated in the mesentery. Van Gieson Hansen. Magnification 800 ×.

Fig. 2. The cellular infiltration and production of connective tissue of an arteriole 48 hours after a primary hypertensive period. The round mononuclear cells are disappearing and the production of connective tissue seems to be slightly increased in comparison with the normal arteriole (Fig. 1). No fibroblast like cells are seen. Carbon particles are deposited in the arteriolar wall. Van Gieson Hansen. Magnification 800 ×.

Fig. 3. The cellular infiltration and production of a viscous matrix of an arteriole 48 hours after a secondary five hours hypertensive period. The infiltrating cells are round and none show a morphology like fibroblasts. A Van Gieson positive matrix without an appearance as fibrils is seen between the cells. Carbon particles are deposited in the arteriolar wall. Van Gieson Hansen. Magnification 800 ×.

Fig. 4. The cellular infiltration and production of connective tissue of an arteriole 48 hours after a secondary hypertensive period with a duration of five hours on each of 4 consecutive days. Many of the cells are typical round cells but others show a morphology like fibroblasts. The production of connective tissue is marked. Van Gieson Hansen. Magnification 800 ×.


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Fig. 3. The cellular infiltration and production of a viscous matrix of an arteriole 48 hours after a secondary five hours hypertensive period. The infiltrating cells are round and none show a morphology like fibroblasts. A Van Gieson positive matrix without an appearance as fibrils is seen between the cells. Carbon particles are deposited in the arteriolar wall. Van Gieson Hansen. Magnification 800 ×.

Fig. 4. The cellular infiltration and production of connective tissue of an arteriole 48 hours after a secondary hypertensive period with a duration of five hours on each of 4 consecutive days. Many of the cells are typical round cells but others show a morphology like fibroblasts. The production of connective tissue is marked. Van Gieson Hansen. Magnification 800 ×.

TABLE 1 *Schematic Representation of Infiltration of Mononuclear Cells and Production of Connective Tissue*

	Viscous matrix	Collagen fibrils	Mononuclear cells	Fibroblast like cells
Normal arteriole	—	+	+	(+)
Primary cellular reaction 48 hours after the hypertensive period	+	+ +	+ +	(+)
Secondary cellular reaction 48 hours after a five hours hypertensive period	+ + + +	+ + +	+ + + +	(+)
Secondary cellular reaction 48 hours after a five hours hypertensive period on each of 4 consecutive days	+ +	+ + + +	+ + +	+ + +
Secondary cellular reaction 48 hours after a five hours hypertensive period in rats pretreated with antithymocyte serum	—	+	+ (+)	(+)

The table shows the correlation between infiltration of mononuclear cells into hypertensively damaged arterioles and production of Van Gieson positive connective tissue

Group 5 (Rats treated with antithymocyteserum) In arterioles from these two rats, the cellular reaction was completely inhibited or only very slight in comparison with the reaction seen in rats not treated with antithymocyteserum The damage of these arterioles estimated on the basis of the arteriolar deposits of carbon particles was identical with the arteriolar damage in rats not treated with antithymocyteserum Corresponding to the sparse cellular reaction no production of connective tissue fibrils or viscous matrix around the damaged arterioles was observed

DISCUSSION

The present results show that there exists a correlation between infiltration of mononuclear cells into hypertensively damaged arterioles and production of connective tissue (Table 1) In the arterioles with a primary mononuclear cellular reaction which was relatively sparse compared with the secondary reaction a production of connective tissue was found which was increased in comparison with normal arterioles but less marked than in the cases with a secondary reaction When the secondary hypertensive period

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more marked than the secondary reaction 24 and 48 hours after the termination of the hypertensive period Corresponding to this varying cellular reaction the production of connective tissue fibrils varied from a very sparse to a very marked amount Corresponding to this it was found that antithymocyte serum pretreated rats had only a sparse cellular reaction and no connective tissue formation The connective tissue showed a morphological appearance like fibrils in most cases but in some preparations the mononuclear cells were situated in a viscous matrix which was stained red with the method of Van Gieson without showing

RESULTS

Group 1 (*Normal rats*) The most suitable area for studying the connective tissue surrounding the arterioles was the mesentery or the area of the small intestine where the arterioles were placed in the tunica muscularis before entering the submucosa, because the natural existence of connective tissue was very sparse in these areas so that it would be easy to estimate an increased production Fig 1 shows the natural existence of connective tissue surrounding a normal arteriole in the mesentery

Group 2 (Rats killed 24 and 48 hours after the termination of the *primary hypertensive* period) 24 and 48 hours after the hypertensive period some mononuclear cells were situated in the connective tissue around the damaged arterioles and in relation to this location of the cells the red Van Gieson Hansen positive fibrils were slightly increased in number in comparison with those seen in normal arterioles (Fig 2) while no difference in the number of fibroblast like cells was seen

Group 3 (Rats killed 24 and 48 hours after the termination of the *second hypertensive* period) Compared with the primary cellular reaction it was characteristic that the secondary cellular reaction was much more marked All the mononuclear cells were round and did not show the morphological characteristics of fibroblasts In relation to this increased cellular reaction an increased amount of Van Gieson positive connective tissue fibrils was observed in many preparations In some preparation it was furthermore observed that the mononuclear cells apparently were situated in a viscous matrix which appeared diffusely red without special organization in fibrils (Fig 3) In some arterioles with carbon particles deposited in the wall a few Van Gieson positive lines were found in the intima and the media

Group 4 (Rats killed after a period of *secondary treatment with angiotensin during three to five consecutive days)* The cellular reaction in arterioles from these rats varied from the sparse reaction observed after a primary treatment with angiotensin to a cellular reaction more marked than that to occur after a single secondary treatment with angiotensin Corresponding to this variation in cellular reaction the production of connective tissue varied proportionally from a slight amount to a very marked amount (Fig 4 and Table 1) The same relation between cellular reaction and production of connective tissue was found by one of my colleges who without any knowledge of the treatment of the rats, was studying the preparations It was characteristic that many cells with a morphology like fibroblasts were situated among the typical round mononuclear cells with a morphology like lymphocytes monocytes and macrophages Many of the round mononuclear cells contained phagocytized carbon particles in their cytoplasm when they were situated corresponding to an arteriole with deposits of carbon and also some of the fibroblast like cells contained carbon particles in their cytoplasm (Fig 5A) It was impossible to estimate whether these fibroblast like cells really produced connective tissue fibrils but it was often found that *fibrils from the two poles* of these spindle shaped cells continued in collagen fibrils Mitoses were never seen in the round mononuclear cells or in the fibroblast like cells In many arterioles Van Gieson positive lines were found in the intima and the media



Fig 5 A A fibroblast like cell with carbon particles in the cytoplasm situated in the adventitia of a damaged arteriole B A round mononuclear cell with carbon particles in the cytoplasm situated in the adventitia of another damaged arteriole Van Gieson Hansen Magnification 1500×

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any morphological structure like fibrils On the basis of these findings it may be concluded that the mononuclear cells, which as previously described (1970a) showed a morphology like lymphocytes and monocytes, have an ability to produce connective tissue fibrils or an unorganized matrix with staining properties like collagen at a time when the cells show no morphological appearance like fibroblasts A production of a viscous fluid in diffusion chambers which contained peritoneal lymphocytes, macrophages, granulocytes and mast cells has earlier been observed by *Shelton & Rice* (1959) when these cells had grown in the chambers for days The curve of absorption of this viscous fluid was like protein and it was suggested that the material was a polymerization of glycoproteins because it was stained with PAS In some preparations from the present study it was furthermore observed that fibroblast-like cells were situated among the round mononuclear cells and that some of the fibroblast like cells contained phagocytized carbon particles in their cytoplasm This seems to indicate that mononuclear cells, after having phagocytized carbon particles in the walls of the damaged arterioles, have been transformed into cells with a morphology like fibroblasts This finding seems to be identical with the observations in diffusion chambers inserted in human beings (*Petrakis et al* 1961) A transformation from lymphocytes and/or monocytes into fibroblast like cells have been observed many times (*Maximow* 1904, 1928, *Ghani* 1968, *Stirling & Kakkar* 1969), while other investigators did not confirm such a transformation (*Hall & Furth* 1938, *Medawar* 1940, *Ebert et al* 1940) The question concerning the possible transformation of mononuclear cells from the blood into fibro blast like cells is still a controversial problem, because the only crucial experiment, namely the direct and constant *in vivo* observation of a transformation of a mononuclear cell (lymphocyte or monocyte) into a fibroblast has not yet been performed

The author is very thankfull to prof *Astrid Fagraeus*, Statens Bakteriologiska Laboratorium, Stockholm, Sweden for supplying the antithymocyteserum and to *P C Eskildsen*, M D for estimation of the connective tissue in the preparations
This work was supported by grants from *Kong Christian X s fond'* and from *'Froken P A Brandts legat*

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test the hypothesis that the inflammatory cellular reaction in the experimental angiotensin induced hypertensive vascular disease is due to hypersensitivity of the delayed type. The test has been performed by injection of lymphocytes from the thoracic duct from previously hypertensively treated rats to normal rats, followed by a single hypertensive treatment of the recipients.

The inflammatory cellular reaction in these rats has been compared with the corresponding cellular reaction in rats which have been injected with lymphocytes from the thoracic duct from non pretreated rats.

MATERIAL AND METHODS

Animals. Inbred female hood rats weighing 150–170 grams.

Anaesthesia. A solution of pentymal sodium 25 mg/ml injected intraperitoneally in a dose of $^1/_2$ ml per 100 grams of rat. A larger dose was used when the rats were killed.

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... hypertensin CIBA dissolved in physiological saline.

The experimental technique. Group 1. Four rats were treated twice at an interval of a week with ...

... as placed in the abdominal section of the thoracic duct and the lymph was drained during the following 48 hours. The drained lymph was injected intravenously to eight normal recipients in a volume of about 2 ml/recipient. Fifteen minutes later hypertension was induced in the recipients using intravenous injections of angiotensin ($^1/_2$–1 microgram every fifth minute during five hours) through a catheter of polyethylene placed in the one femoral vein. At the termination of the hypertensive period 0.1 ml of colloidal carbon particles was injected intravenously through a catheter in the other femoral vein. The rats were killed 24, 48, 72 and 96 hours after the termination of the hypertensive period. The small intestines were fixed in a 4 per cent solution of buffered formalin. Mesenteric arterioles and arterioles situated in the submucosa of the small intestine with colloidal carbon particles deposited in the walls were isolated, embedded in paraffin, cut in five micron thick serial sections and stained with haematoxylin-eosin. From each rat about 13 arterioles were isolated and about 25 serial sections from each arteriole studied under the microscope.

Group 2. In three normal rats a polyethylene catheter was placed in the abdominal section of the thoracic duct and the lymph was drained during the following 48 hours. The drained lymphocytes were injected intravenously to five recipients which thereafter were treated with intravenous injections of angiotensin and colloidal carbon particles at the same times and the tissue treated as described above under group 1.

RESULTS

Group 1. One of the eight rats which received lymphocytes from the thoracic duct from rats previously treated twice with acute hypertension died at the termination of the hypertensive period and three other rats died in the course of the following six hours after the hypertensive period. Examination of their small intestines under a stereo microscope showed a very marked deposition of colloidal carbon particles in the walls of both arterioles and venules indicating a very severe penetration of plasma components into the vessel walls during the hypertensive period. The remaining four recipients were killed 24, 48, 72 and 96 hours after the termination of the hypertensive period. In these rats it was also found that colloidal carbon particles were deposited in the walls of both arterioles and venules but the deposition specially in the venules was less marked than in the rats which died in the hours following the termination of the hypertensive period.

The damaged arterioles showed an inflammatory cellular reaction which was in progression in the adventitia from the beginning of the examination of the cellular reaction and to the termination 96 hours after the hypertensive period. In the lumen and the media the cellular reaction was nearly constant during the period of the examination.

The majority of the cells situated in the adventitia were mononuclear with a morphology like small lymphocytes, large lymphocytes, monocytes and macrophages ...

... was estimated counting

EVIDENCE FOR AN IMMUNOLOGICAL FACTOR IN THE HYPERTENSIVE VASCULAR DISEASE

FINN OLSEN

The University Institute for Experimental Medicine (Head Jens Bing, M.D.)
Copenhagen, Denmark

Hypertensive vascular disease was studied in acute experimental angiotensin induced hypertension. If the hypertension was induced in rats pretreated with injection of thoracic duct lymphocytes the quantity of the mononuclear inflammatory cellular reaction in the damaged arterioles was 2–9 times higher and more prolonged than in control rats. This difference in the cellular reaction is in agreement with the cellular reaction in cases of hypersensitivity of the delayed type.

Hypertensive vascular disease is characterized by degeneration increasing to necrosis of the arteriolar walls and infiltration with inflammatory cells. The genesis of the inflammatory cellular reaction has been obscure but it seems likely that an immunological mechanism of delayed type is involved.

Substances with qualities like antigens can be isolated from the tunica intima of aorta (*Intorp & Milgrom* 1969). Rats injected intraperitoneally with extracts of homologous aorta produce antibodies towards unknown antigens of the extracts, which can be demonstrated in the serum using a precipitation reaction. The existence of circulating antibodies in the rats is accompanied by a degeneration of and an inflammatory cellular infiltration into the mesenteric arterioles (*White & Grollman* 1964).

If hypertensive rats are treated with methods of immunosuppression, the frequency of the arteriolar damage is decreased (*Gardner et al.* 1970). Some patients with severe hypertension have a positive complement consumption test (*Köröskényi et al.* 1961) or increased values of immunoglobulins in the serum (*Doyle & Ebringer* 1970) which might be an index of hypertensive vascular injury.

Rats treated with a period of acute experimental angiotensin induced hypertension of about five hours show in the following days morphological changes of both the mesenteric arterioles and their regional lymph nodes composed of a proliferation of large pyroninophilic cells (immunoblasts) followed by a proliferation of small pyronine-negative cells with a morphology like lymphocytes (*Olsen* 1970a). If a secondary hypertensive period is induced the secondary inflammatory cellular reaction is prolonged and 3–10 times more pronounced than the primary reaction (*Olsen* 1970b), which is a course like the cellular reaction in cases of hypersensitivity of the delayed type (*Kosunen et al.* 1963).

The aim of the present work has been to

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the number of mononuclear cells in the lumen the media and the adventitia of five consecutive serial sections of arterioles showing deposition of colloidal carbon particles in the wall The quantitative distribution of these cells 24 48 72 and 96 hours after the termination of the hypertensive period is seen in Table 1A The most marked inflammatory cellular reaction was found after 96 hours (Fig 1A)

Group 2 (Control rats) None of the rats in this group died during the hypertensive period or in the hours thereafter The rats were killed 24 48 48 72 and 96 hours after the termination of the hypertensive period They showed deposition of colloidal carbon particles in arteriolar but not in venular walls in the mesentery or the submucosa of the small intestine in a degree which was about the same as the arteriolar deposition in group 1

Examination of tissue sections from these rats showed an inflammatory cellular reaction which was maximal in the tunica media after 48 hours and in the adventitia after 24 and 48 hours disappearing during the following two days The mononuclear cellular reaction was composed of cells with a morphology like lymphocytes and monocytes Only very seldom a polymorphous leucocyte was observed

The quantitative distribution of the mononuclear cells was determined as in group 1 and the result is seen in Table 1B

Fig 1B shows the cellular infiltration in a damaged arteriole from a control rat 96 hours after the termination of the hypertensive period It is seen that the cellular reaction in this vessel is markedly less than in Fig 1A

DISCUSSION

The results in Table 1A and 1B show that the course and the quantity of the mononuclear cellular infiltration in hypertensively damaged arterioles from rats injected intravenously with lymphocytes from rats treated with acute hypertension are prolonged and 2 9 times higher than the corresponding cellular reaction in arterioles from rats injected with lymphocytes from *normal* rats when arterioles with about the same degree of hypertensive damage are compared A difference in the quality of the cellular infiltration was also found as polymorphous leucocytes were found among the mononuclear cells in the adventitia 72 and 96 hours after the termination of the hypertensive period in group 1 but not in group 2

This means that the previously mentioned hypothesis (*Olsen 1970b*) *concerning a hypersensitivity of the delayed type in the hypertensive vascular disease has been proven in these transfer experiments with thoracic duct lymphocytes*

The question remains unanswered, which role such hypersensitivity plays in the development of necrosis of the walls of the hypertensively damaged arterioles But the studies by *Gardner et al* (1970) show that an immunological mechanism might influence the development of degeneration of the arteriolar walls in hypertensive rats

The very marked deposition of colloidal carbon particles in the *venular walls* in the mesentery and the submucosa of the small intestine observed in the rats injected with lymphocytes from previously hypertensively treated rats might be a result of an antigen antibody reaction followed by a severe increased permeability of plasma components In a previous study (*Olsen* 1968) it was found that fluorescent proteins became deposited in the venular walls during an acute angiotensin hypertensive period which shows that also the venules are damaged in this experimental model for studying hypertensive vascular disease This means that a possibility exists for an antigen antibody reaction in the venules as a result of the immunological reaction.

The author is very thankfull to *H L Ford* M D and *P E Nielsen* M D The University Institute for Experimental Immunology Copenhagen for supplying the thorac c duct lymphocytes

This work was supported by grants from *Kong Christian X s fond* and from *Frøken P A Brandts legat*

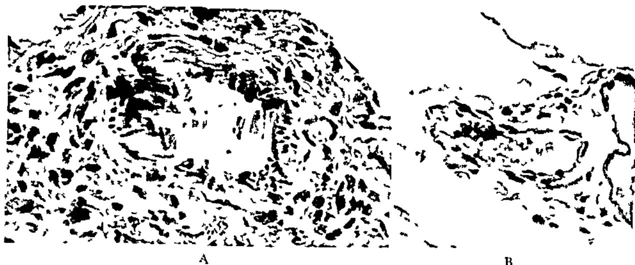


Fig 1A Mesenteric arteriole with deposition of colloidal carbon particles in the wall from a rat to which thoracic duct lymphocytes first were transferred from a rat previously treated with acute hypertension and fifteen minutes later treated with injections of angiotensin during a period of five hours The rat was killed 96 hours after the termination of the hypertensive period No mononuclear cells are sticking to the damaged endothelium, a few are situated in the tunica media, but the majority of the mononuclear cells are situated in the adventitia in which the cellular reaction is very marked in comparison with that in the control rat (Fig 1B) Haematoxylin eosin Magnification 400 ×

Fig 1B Mesenteric arteriole with deposition of colloidal carbon particles in the wall from a rat to which thoracic duct lymphocytes were transferred from a non pretreated rat, 96 hours after the termination of the hypertensive period No cellular reaction is seen in the lumen and the tunica media The cellular components in the adventitia are sparse as in normal rats Haematoxylin eosin Magnification 400 ×

TABLE 1A *The Numerical Distribution of the Mononuclear Cells in the Lumen, the Media and the Adventitia*

Time in hours (h) after termination of the hypertensive period	lumen	media	adventitia
24 h	75	52	412
48 h	79	43	428
72 h	62	25	588
96 h	73	45	934

TABLE 1B

Time in hours (h) after termination of the hypertensive period	lumen	media	adventitia
24 h	27	4	210
48 h	40	12	202
72 h	30	7	179
96 h	25	0	101

The numerical distribution of the mononuclear cells in the lumen the media and the adventitia in five consecutive serial sections of ten arterioles from experiments with lymphocyte transfers from rats previously treated with acute hypertension (A), and from the control experiments (B) The quantity of the cellular infiltration in Table 1A is 2–9 times higher than the corresponding cellular reaction in Table 1B at any time after the termination of the hypertensive period

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Acta path microbiol scand Section A 79, 27–31, 1971

CORRELATION OF HISTOLOGIC FEATURES IN TWO GROUPS OF LIVER BIOPSIES WITH CIRRHOSIS AND FATTY CHANGE THE FIRST WITH AND THE SECOND WITHOUT MALLORY BODIES

PER CHRISTOFFERSEN and HEMMING POULSEN

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On comparison of histological qualities in two consecutive series *of liver biopsies all exhibiting* cirrhosis and fatty change, one with and one without Mallory bodies the morphological activity (measured by the number of *disintegrated liver cells and* [illegible] [illegible] is more pronounced in biopsies with Mallory bodies The possible reasons for this difference are discussed

While the occurrence of Mallory bodies in cirrhosis of the liver by the majority of workers (a o *Baggenstoss & Stauffer* 1952, *Popper* 1961) is regarded as an expression for the activity and progression of the pathological process no comparative examinations of consecutive materials exist to the best of the authors knowledge, which unambiguously show whether Mallory bodies are an expression of such activity or not

The purpose of the work presented here has been to compare histological features in two consecutive series of liver biopsies all exhibiting cirrhosis and fatty change, the first with the second without Mallory bodies

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MATERIAL AND METHODS

The material consists of a total of 120 percutaneous liver biopsies all exhibiting cirrhosis (*nodular regeneration* and fibrosis) and fatty change

The material has been selected as consecutive biopsies showing *cirrhosis* and fatty change from a total of 1 100 percutaneous liver biopsies (*Christoffersen* 1970) received at the Pathological Anatomical Institute at the *Kommunehospital* during the period October 1st 1965 to October 20th 1967

Nineteen biopsies have been performed by the Vim Silverman method, while the remaining have been performed by the Menghini method The biopsies have been received from six medical departments (*Copenhagen Study Group for Liver Diseases* 1969) They are 1–1 4 mm thick and 1.5–4 5 cm long

The tissue has been fixed in neutral formalin, embedded in paraffin and sectioned From the first 45 biopsies 10–15 sections were cut on a sledge microtome, while approximately 40 sections were cut on a rotary microtome from the last 75 biop

TABLE 1 *Frequency of a Number of Histologic*

	Liver cell necrosis		Acidophilic bodies		Degree of fatty infiltrat	
	0	+	0	+	+	++
Group 1 (45 biopsies with Mallory bodies)	2 (4 %)	43 (96 %)	8 (18 %)	37 (82 %)	19 (42 %)	23 (51 %
Group 2 (75 biopsies without Mallory bodies)	22 (29 %)	53 (71 %)	38 (51 %)	37 (49 %)	49 (65 %)	17 (23 %
Differences (2 test)	$p < 0.001$		$p < 0.001$		$50 > p >$	

The figures indicate the number of biopsies in each subgroup of the seven qualities. The percentage which the number constitutes of the subgroup is given in brackets.

sies. The sections are approximately 6 µm in thickness.

The assessment has been performed without any knowledge of the clinical data by the two authors in close cooperation on haematoxylin and eosin and v. Gieson Hansen preparations. Furthermore sections stained for reticulin (*Gomori* 1937), iron (*Perls* 1867) and pyroninophil substance (*Brachet* 1942) have been available from all biopsies.

The following qualities have been registered: occurrence of Mallory bodies, alcoholic hepatitis (*Christoffersen et al.* 1970), focal lytic necroses (liver cell necroses excluding necroses in alcoholic hepatitis and lipogranulomas), acidophil bodies, Kupffer cell proliferation, cholestasis (incontrovertible intra- or extracellular bile thrombi), lipogranulomas (*Christoffersen et al.* 1971), parenchymal inflammation, bile duct proliferation and inflammation of the portal tracts, as well as iron deposits in liver cells, Kupffer cells and connective tissue. Further the degree of fatty change and fibrosis (1–3), as well as whether the normal architecture has been completely or partly destroyed have been registered.

The material is divided into two groups: *Group 1* containing Mallory bodies and *group 2* without. The histological features of the two groups have been compared.

For statistical evaluation the chi² test has been used. The limit for type 1—error (2 α) has been set at 0.05.

RESULTS

In the 120 biopsies with cirrhosis and fatty change Mallory bodies were found in 45 (group 1), while 75 (group 2) were without.

Distribution according to sex and age shows no statistically significant differences between group 1 and 2. The average age for the total material is 59 years (range 32–86 years), and 80 per cent are men.

Thirtyfour (76 per cent) of the biopsies in group 1 exhibit changes as seen in alcoholic hepatitis (necroses of liver cells containing Mallory bodies with infiltration of neutrophils).

A comparison of the histologic features in the two groups shows a number of statistically significant differences of which the majority are stated in Table 1.

Lytic liver cell necroses. Focal lytic necroses are observed in the majority of biopsies from both groups, but there are significantly more biopsies with necroses in group 1 (43 (96 per cent)) than in group 2 (53 (71 per cent)) ($p < 0.001$). Confluent necroses have not been demonstrated.

Acidophil bodies. Something similar to the above is the case here, as significantly more biopsies with acidophil bodies are found in group 1 (37 (82 per cent)) than in group 2 (37 (49 per cent)) ($p < 0.001$).

Fatty change and lipogranulomas. There is no statistically significant difference in the degree of fatty change between the two groups, but the number of biopsies with lipogranulomas is significantly greater in group 1 than in group 2 ($p < 0.001$).

Mesenchymal reaction in the parenchyma. Both the incidence of Kupffer cell proliferation ($p < 0.02$) and of parenchymal inflammatory cells ($p < 0.001$) are greater in group 1 than in group 2.

ities in Group 1 and Group 2 of the Material

	Lipogranulomas		Kupffer cell proliferation		Parenchymal inflammation		Degree of destroyed architecture	
+ +	0	+	0	+	0	+	subtotal	total
%)	13 (29 %)	32 (71 %)	0 (0 %)	45 (100 %)	3 (7 %)	42 (93 %)	19 (42 %)	26 (58 %)
%)	47 (63 %)	28 (37 %)	8 (11 %)	67 (89 %)	48 (64 %)	27 (36 %)	46 (61 %)	29 (39 %)
	$p < 0.001$		$p < 0.02$		$p < 0.001$		$p < 0.05$	

Lymphocytes are encountered frequently in both groups (group 1 30/45, group 2 24/75) while neutrophils are much more frequently seen in group 1 (35/45 against 4/75 in group 2) Plasma cells have only been demonstrated in a total of three biopsies (one from group 1, two from group 2)

Degree of destroyed architecture Relatively more biopsies from group 1 than from group 2 exhibit a total destruction of the normal lobular architecture, and the difference is significant ($p < 0.05$), though less pronounced than the above stated correlations

Portal inflammation While there is no difference in the incidence of portal inflammation in the two groups as all biopsies, save two from group 2, exhibit portal inflammation, there are significantly more biopsies in group 1 with neutrophils (34) than in group 2 (11) ($p < 0.001$) However, there is no difference in the incidence of the other inflammatory cells in the two groups Lymphocytes and macrophages are found in all biopsies from group 1 and in 72 from group 2, while the number of biopsies with plasma cells are 34 and 37, and with eosinophils seven and three.

Bile duct proliferation There is bile duct proliferation in nearly all biopsies, and there is no difference between the two groups

Fibrosis In the greater majority of cases there is a moderate or severe fibrosis, and there is no difference between the two groups

Cholestasis and iron deposits Only a minority of the biopsies exhibit cholestasis and iron deposits. There is no difference between the two groups

To summarize there is a greater incidence of focal liver-cell necroses and acidophil bodies, and more pronounced inflammation, as well as a greater degree of destroyed lobular architecture in biopsies with Mallory bodies than in biopsies without Mallory bodies, while there is no decisive difference in the degree of fatty change and fibrosis or the incidence of bile duct proliferation, cholestasis and iron

DISCUSSION

In cirrhosis with fatty change one may in the light microscope observe liver cells disintegrating in the following manners lytic liver cell necroses, acidophil bodies lipogranulomas, and necroses in alcoholic hepatitis If the histological activity in a cirrhosis is expressed by the amount of disintegrated liver cells and the inflammatory reaction brought about by these, there is in the presented material a far greater activity in the group of biopsies with Mallory bodies than in the group without This is the case even when the necroses which are part of the alcoholic hepatitis and which are found in 76 per cent of the biopsies containing Mallory bodies are disregarded

It is thus possible to speak about two morphologically different kinds of activity in liver tissue with cirrhosis and fatty change, namely alcoholic hepatitis and the other kinds of

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liver cell necroses with mesenchymal reaction

Lytic necroses, acidophil bodies and lipogranulomas are found diffusely distributed throughout the liver tissue and without any particular morphologic relation to the areas, where alcoholic hepatitis is to be found While it must be presumed that the Mallory bodies have a direct influence on the development of alcoholic hepatitis, the morphological appearence suggests that it is not the Mallory bodies as such which produce the remaining activity However, as there is a considerable degree of positive correlation between the incidence of Mallory bodies and the other signs of activity, it may be said with certainty, that there is a temporal connection and possibly also an aetiological

While there is no certain difference in the degree of cirrhosis as gauged by portal and septal fibrosis in the two groups, the degree of destroyed architecture is greater in the group with Mallory bodies

The more frequent incidence of greater histologic activity and more indistinct lobular architecture in the group with Mallory bodies speaks, when related to the fact, that it has previously been shown, that parenchymal fibrosis is more pronounced in biopsies with fatty change (without cirrhosis) and Mallory bodies than in biopsies with fatty change (without cirrhosis) and no Mallory bodies (*Christoffersen & Juhl* 1970), in favour of a causal relation between Mallory bodies and development and progression of cirrhosis In this case it would be reasonable to suppose that the necroses of the liver cells and the accompanying inflammatory process, which occurs with particular strength in liver tissue containing Mallory bodies, is the cause or a contributary cause for the devclopment of the nodular regeneration How great a part the alcoholic hepatitis and the remaining activity plays for the development of nodular regeneration cannot be said on the basis of the present material, but previous investigations have shown that small lytic liver cell necroses, such as are in question here, and acidophil bodies play no part in the development of parenchymal fibrosis in livers exhibiting fatty change (*Christoffersen et al* 1970) However, there is a strong presumption that the rather rarely occurring lipogranulomas type III in some cases may bring about the development of connective tissue in the lobuli (*Christoffersen et al* 1970)

According to the available facts *it is thus very likely, that alcoholic hepatitis is the common cause for development of cirrhosis in livers exhibiting fatty change (and for progression of the cirrhosis)*, but lipogranulomas type III *may also play a part*

Rice & Yesner (1960) in follow up examinations find that occurrence of Mallory bodies in liver biopsies is accompained by both morphological and clinical activity This is, when group 1 is considered in its entirety, in agreement with our results, though it must be noted that we in a few cases have found cirrhosis exhibiting fatty change and containing Mallory bodies with practically no morphological activity

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compatible with preservation of the metachromatic stainability and morphological integrity of the mast cells

MATERIAL AND METHODS

The experimental animals consisted of (22) Spraque Dawley rats of both sexes ranging in weight from 90 to 120 g The right lower leg of each animal was fractured manually under ether anaesthesia and the fractures allowed to heal until the animals were killed after 1 2 3 4 and 5 weeks respectively (Fig 1 A) 24 hours before sacrifice the rats were injected intraperitoneally with 4 μCi/g body weight of ^{35}S in 0 2 ml of a 0 5 per cent sterile aqueous solution of sodium sulphate At autopsy specimens for histological examination were taken from the periosteal callus (Fig 1 B) excluding any fragments of broken bone and fixed for 48 hours in a 4 per cent aqueous solution of lead subacetate The specimens were then rinsed overnight in running tap water dehydrated embedded in paraffin wax and sectioned at 5 7 μm (*Lindholm S* 1959) Specimens from the tissues surrounding the left unfractured lower leg were also taken and treated as above

From the sections autoradiographs were prepared on Kodak AR 10 emulsion by stripping film technique (*Pelc* 1947, *Rogers* 1967) The films were exposed in a dry atmosphere at +4° C for 17 days and processed according to Kodak scientific and technical data sheet P 64 After the final wash the preparations were stained for 5 minutes in Unnas polychrome blue dried in air and mounted The photomicrographs were focused on a) the mast cells in the tissue section b) the silver grains of the overlying emulsion To ensure that the darkening of the emulsion seen above the mast cells was not due to a chemographic artefact the legs of a number of rats that were not previously given ^{35}S were also broken and the ensuing callus treated as above

The mean grain count per mast cell in the course of the process of repair was calculated by weekly counts of the grains above five equally large mast cells in the fibrous callus

RESULTS

Microscopic observations of the cells Metachromatic granular mast cells (Fig 1 C) were observed in the fibrous callus in all sections every week in which they were examined after fracture In the peripheral regions of the callus formation at some distance from the cartilage and bone, the cells were of the so-called "well fed" character, while the morphology of those near the cartilaginous zone changed in the direction of disintegration and degranulation The metachromatic colour of the ground substance generally increased in intensity towards the cartilage zone and reached a maximum in the walls between the cartilage cells In the preparations made from tissues surrounding unfractured bone, mast cells of normal granulation were observed in periosteum, muscle and connective tissue

Microscopic observations of the autoradiographic emulsion A dense aggregation of silver grains over the metachromatically staining granules in the mast cells in the fibrous parts of the callus was noted in all sections (Fig 1 D), which points to an accumulation of ^{35}S labelled compounds Especially in sections from specimens taken during the first two weeks after fracture this aggregation was evident A somewhat more pronounced concentration of the uptake per cell was observed in the peripheral regions, while in closer proximity to the cartilage zone a wider and less dense distribution of labelled material was seen which partly involved even the ground substance between the cells [illegible] in the mast cells and in the tissues surrounding unfractured bone was noted

Quantitatively examined, the mast cells seemed to take up the largest amounts of ^{35}S label during the first two weeks after fracture, as the grain count per cell reached a maximum at this time During the following 3–5 weeks when the number of mast cells in the callus has been shown to diminish (*Lindholm, R et al* 1967), the grain count per cell was distinctly lower and of the same order as the counts of mast cells found in tissues surrounding unfractured bone Autoradiographically, the callus tissue as a whole showed moderate or slight uptake of ^{35}S when the mineralization was in progress In Fig 2 the mean grain counts per

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AUTORADIOGRAPHIC DEMONSTRATION OF SULPHATED MUCOPOLYSACCHARIDES IN FIBROUS CALLUS MAST CELLS IN FRACTURE REPAIR IN RATS

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Mast cells in the periosteal callus of healing experimental fractures in rats were shown to incorporate ^{35}S labelled substances During the first two weeks after fracture, *i e* the organic phase of repair, the uptake of label reached a maximum As mineralization of the fractures proceeded, the labelling became less intense and the total number of mast cells decreased It is discussed whether the mucopolysaccharides of the mast cells undergo metabolic changes in the process of repair

Sulphated mucopolysaccharides are present in the ground substance of normal bone as well as in fracture callus (*Balazs* & *Rogers* 1965) Bone and cartilage are free of mast cells, although mast cells occur in the connective tissue surrounding these structures (*Urist* & *McLean* 1957) It has been noted that mast cells accumulate in the fibrous parts of the fracture callus (*Lindholm, R et al* 1967) The probable existence of a close connection between polysaccharides and the initiation of mineralization has been suggested (*e g Bowness* 1968) The functional connection between connective tissue mast cells and sulpho polysaccharides has been demonstrated in studies on the incorporation of ^{35}S (*Jorpes et al* 1953 *Asboe Hansen* 1954) Mast cells presumably provide the regenerating bone with substances *necessary for maturation and mineralization* (*Lindholm, R et al* 1969)

A great number of studies concerning fracture repair using radioactive isotopes are to be found in the previous literature (*e g Duthie* & *Barker* 1955 *Singh* & *Udupa* 1964, *Koskinen* 1965) No evidence of the uptake of ^{35}S by mast cells in fracture callus has hitherto been reported *despite the publi*cation of detailed observations of the incorporation of this isotope in the growing epiphyses proximal and distal to the fracture (*Duthie* & *Barker* 1955) This report relates an attempt to label mast cells in the periosteal callus with ^{35}S, using a histotechnique

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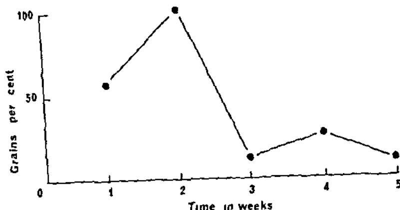


Fig 2 Relative grain counts from autoradiographs of callus mast cells from healing fractures of rats

cation reorganization and resorption of the callus take place (*Solheim* 1965)

An experimentally fractured leg exhibited greater accumulation of ³⁵S than the intact leg and a pronounced maximum appeared on the 7th to 9th day after fracture (*McDonald et al* 1957) It has been demonstrated (*Dziewiatkowski* 1951) that radiosulphur shows a particular predilection for the cartilage and is incorporated in organic form as chondroitin sulphate The process of fracture repair is apparently related to the synthesis of chondroitin sulphate in the newly formed collagen tissue (*Kowalewski* 1958) The largest amounts of chondroitin sulphates was observed on the 11th day when also the metachromasia of the callus tissue reached a maximum The accumulation of minerals occurred concomitantly with the disappearance of uronic acids hexosamines and metachromasia (*Penttinen* & *Kulonen* 1970)

Mast cells in the skin take up ³⁵S but a considerable proportion of them do not (*Asboe Hansen* 1954) even in tissue with strong mast cell activity In this autoradiographic study it was observed that the mast cells showed heavy labelling with ³⁵S in the organic phase of repair The cellular activity, which utilized increasing amounts of the circulating isotope for synthesis of acid mucopolysaccharide components for the fibro cartilaginous callus has at this time reached a maximum Later on the number of labelled mast cells is evidently smaller and the grain counts have also diminished Probably, in mast cells there may be sulphated and/or non sulphated mucopolysaccharides of the heparin and hyaluronic acid types (*Asboe Hansen* 1954) Apparently the mucopolysaccharides in the organic phase of repair are of the sulphated type and later on the hyaluronic acid type may be the chief polysaccharide exhibited Biochemically it has been demonstrated (*Solheim* 1965) that hyaluronic acid chondroitin 4 sulphate and chondroitin 6 sulphate increased during the first two weeks after fracture and then declined Peritoneal mast cells investigated by column chromatography were shown to contain chondroitin 4 sulphate, chondroitin 6 sulphate, dermatan sulphate and heparin (*Horsfield et al* 1966)

In the mineral phase of repair, the reduced uptake of the isotope in the granules indicates that less sulphated substances are being formed It is apparent that the ability of the mast cells in the callus to incorporate radiosulphur depends on their metabolic state and that the sulphuric acid content of the cells varies during the course of fracture repair

In the process of biological calcification in general mast cells have been shown to transport calcium ions to sites where hydroxy

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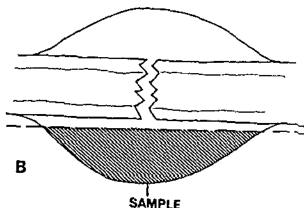






Fig 1 Data on 15 days old experimental fracture of the tibia of a rat

A X ray of fracture

B Localization of sample for histology

C Section through periosteal callus showing aggregation of metachromatic mast cells (Unnas polychrome blue 800 ×)

D Same preparation focused on the autoradiographic emulsion Mast cells show incorporation of ^{35}S label

cell are presented graphically as a function of the time in weeks after fracture The maximum count is noted as 100 and the others in per cent of this

DISCUSSION

The process of fracture healing can be divided into an organic phase which lasts for about two weeks after injury and a mineral phase during which rapid deposition of mineral around the collagen fibres takes place (*Singh* & *Udupa* 1964) It has been noted that an increase in the hexosamines of normal fracture callus reaches a maximum about two weeks after the fracture and then decreases (*Bolognani* 1961 *Penttinen* & *Kulonen* 1969) The hexosamine content is regarded as an index to the amounts of mucopolysaccharides present and these probably begin to decrease slowly when calcifi

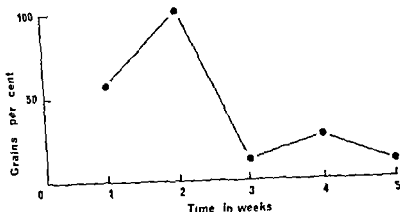


Fig 2 Relative grain counts from autoradiographs of callus mast cells from healing fractures of rats

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Mast cells in the skin take up ^{35}S, but a considerable proportion of them do not (*Asboe Hansen* 1954) even in tissue with strong mast cell activity In this autoradiographic study it was observed that the mast cells showed heavy labelling with ^{35}S in the organic phase of repair The cellular activity, which utilized increasing amounts of the circulating isotope for synthesis of acid mucopolysaccharide components for the fibrocartilaginous callus, has at this time reached a maximum Later on, the number of labelled mast cells is evidently smaller and the grain counts have also diminished Probably, in mast cells there may be sulphated and/or non sulphated mucopolysaccharides of the heparin and hyaluronic acid types (*Asboe-Hansen* 1954) Apparently, the mucopolysaccharides in the organic phase of repair are of the sulphated type and later on, the hyaluronic acid type may be the chief polysaccharide exhibited Biochemically it has been demonstrated (*Solheim* 1965) that hyaluronic acid, chondroitin 4 sulphate and chondroitin 6 sulphate increased during the first two weeks after fracture and then declined Peritoneal mast cells investigated by column chromatography were shown to contain chondroitin 4 sulphate, chondroitin 6 sulphate, dermatan sulphate and heparin (*Horsfield et al* 1966)

In the mineral phase of repair, the reduced uptake of the isotope in the granules indicates that less sulphated substances are being formed It is apparent that the ability of the mast cells in the callus to incorporate radiosulphur depends on their metabolic state and that the sulphuric acid content of the cells varies during the course of fracture repair

In the process of biological calcification in general, mast cells have been shown to transport calcium ions to sites where hydroxy-

3*

apatite is being formed in the tissues (*Selye et al* 1964, 1965). It has been shown by atomic absorption spectrophotometry (*Phil et al* 1967) that isolated peritoneal mast cell granules contains zinc and iron, but not copper and calcium The zinc probably stabilizes a heparin-histamine complex in the granules It has not yet been studied whether mast cell degranulation is related to the precipitation of calcium in the callus of healing fractures

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Acta path microbiol scand Section A 79 37-42 1971

THE THYMUS IN INFANTS DEAD FROM ACUTE DISORDERS

Thymus Weight and Cortico Medullary Ratio

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Thymus weight and cortico-medullary size ratio were studied at autopsy in 49 infants between two weeks and two years and a half of age who died either from sudden unexpected and unexplained death (SUD) or from accidents or other explained non infectious causes (ACC) or from acute infections of less than two weeks duration (ILL) Thymus weight was slightly lower in the SUD group than in the ACC group Approximate normal curves for absolute and relative thymus weights and for cortico-medullary ratio as estimated from planimetric measurements on histological sections were drawn on the basis of the combined SUD and ACC groups Thymus weights and ratios in the ILL group were expressed as per cent of the normal curves and related to the duration of disease Cortico-medullary size ratio showed less individual variation than thymus weight and may give a more useful estimate of the functional state of the organ When disease has lasted for from one to two days thymus weight and cortico-medullary ratio may or may not be within normal range When disease has lasted for more than two days the values are below normal range Little or no significant deviation from normal can be expected when disease has lasted for less than 8-12 hours

The concept of status thymico-lymphaticus as a disorder or anomaly predisposing to sudden death in otherwise healthy infants took origin from the observation that infants who die suddenly have much larger thymuses than infants who die from disorders that have been observed for some time before death The concept was generally abandoned when it was shown that the thymus is not larger in sudden unexpected and unexplained death (SUD) than in sudden deaths from explained causes (5 11 13) In experimental animals thymic involution can occur very rapidly as a result of many kinds of stress (6 25) The large thymus in SUD is now commonly believed to represent the normal state, and it may even be argued that the large thymuses speak against any hypothesis of SUD that implicates a period of disease prior to death It is only incompletely known however, how many hours or days a state of illness must have been present in human infants to produce a significant reduction in thymus weight Boyd (5) stated that when illness has lasted for more than 24 hours the weight of the thymus is reduced regardless of the cause of death However, the normal standard in her analysis was derived from the group of infants who died after 0-24 hours of illness and this group as a whole was compared to the broad group of infants who died after one to seven days of illness

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Even in groups of individuals in whom no disease has been present prior to death, the weight of the thymus shows a large individual variation. This is seen in infants who die suddenly from accidents or homicide (13), as well as in normal laboratory animals (1, 25). There is good reason, therefore, to look for other parameters that can give more consistent measures of the functional state of the thymus than the total weight of the organ.

The present study shows thymus weights in infants who had been considered to be healthy until they were found dead, and who died either from explained non-infectious causes or as cases of SUD. The findings are compared to findings in infants who died from observed acute diseases. In the latter cases the data on the thymus are related to the duration of disease. In addition to thymus weight, the ratio between cortical and medullary volumes and the ratio of the density of the lymphocyte population in the cortex and the medulla have been estimated.

MATERIAL

The study is based upon 49 infants between two weeks and two years and a half of age who died from acute disorders and were autopsied at Ullevål Hospital during the period 1959-1968. Both thymus weight and histological sections from the thymus were available in 30 cases, sections only in 14 cases, and thymus weight only in 5 cases. All except three cases were included in a previous study on SUD (20).

The material has been divided into three groups for which abbreviated designations will be used:

SUD – Sudden unexpected and unexplained death. The group comprises 15 boys and 7 girls who were unexpectedly found dead in bed or pram without having shown evidence of disease other than a trivial cold, and in whom autopsy findings were inconclusive.

ACC – Sudden deaths from accidents and other non-infectious explained causes. 6 boys and 3 girls. Six infants died from accidental suffocation, one died from acute pontine and cerebellar haemorrhage, one died suddenly from endocardial fibro-elastosis, one died three hours after an injury to the head.

ILL – Deaths from acute disorders of less than two weeks duration. 12 boys and 6 girls. Seven infants died from acute gastro-enteritis after $^1/_2$, $1^3/_4$, $1^3/_4$, 2, 3, 3 and 5 days; six died from pneumonia or acute tracheobronchitis after $^3/_4$, 1, $1^1/_4$, 5, 5 and 9 days; two died from high fever and convulsions after $^3/_4$ and $1^1/_2$ days; one died from myocarditis and a probably viral pneumonia after 7 days; one died from combustion after 2 days; one died 2 days after an injury to head.

All infants had been considered healthy until the onset of the actual acute disorder.

METHODS

Thymus and body weights were taken from autopsy records. Histological sections were examined without knowledge of which group each specimen belonged to. For the determination of the relative size of cortex and medulla, thymus sections were projected onto a sheet of paper at a magnification of 16 ×. In an area corresponding to 0.2 – 0.7 cm² section area, the outlines of cortex and medulla were traced on the paper, and their respective areas were measured with a planimeter (9). The numbers of lymphocytes (small and medium sized) per unit section area in the cortex and in the medulla of one representative lobule were determined by means of an eye-piece reticle.

RESULTS

The absolute weight of the thymus is shown in Fig. 1. An average weight curve for the combined SUD and ACC groups has been drawn by eye. Thymus weight in these groups increases rapidly during the first three months of life and then levels off at an average of about 30 grams.

Thymus weight relative to body weight is shown in Fig. 2. The relative thymus weight increases to a peak in the third month of life and decreases gradually thereafter. An average curve for the combined SUD and ACC groups has been drawn be eye as in Fig. 1.

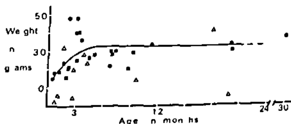


Fig. 1. Absolute weight of the thymus. Closed squares: SUD group; closed circles: ACC group; open triangles: ILL group. The curve line refers to the combined SUD and ACC groups.

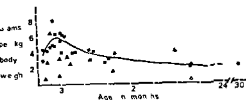


Fig 2 Relative weight of the thymus Symbols as in Fig 1 The curve line refers to the combined SUD and ACC groups

The aim of drawing these curves was to produce on the basis of the combined groups of sudden death in previously healthy infants normal reference lines to which thymus weights of the ILL group could be compared. The curves are referred to as normal weight curves

When the individual SUD and ACC plots are compared to these normal weight curves it appears that the majority of ACC values are above the line and the majority of SUD values are below the line This is seen for both absolute and relative thymus weights

Thymus weights in the ILL group are mostly well below the normal weight curves but there is some overlap of individual values (Figs 1 and 2) Figs 3 and 4 show the relation between thymus weight and the dura

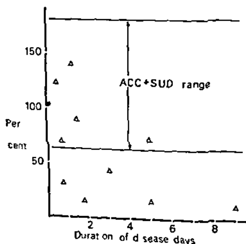


Fig 3 Absolute thymus weight expressed as per cent of the SUD + ACC curve in Fig 1 and related to the duration of illness Open triangles ILL group closed circle infant in the ACC group who lived for three hours after injury to the head

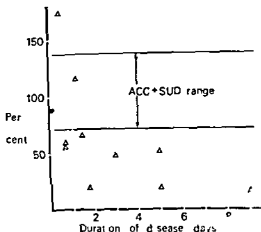


Fig 4 Relative thymus weight expressed as per cent of the SUD + ACC curve in Fig 2 and related to the duration of illness Symbols as in Fig 3

tion of illness in the ILL group Thymus weights are given in per cent of the normal weight curves derived in Figs 1 and 2 The shortest duration of disease in which thymus weight is below normal range is 3/4 day, and most weights are below normal range when disease has lasted for two days or more

The separate weights of the cortex and the medulla were calculated from the total thymus weight and the planimetric estimates of the

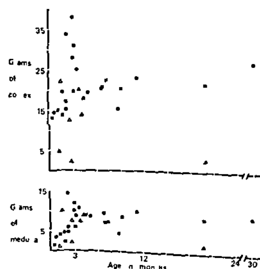


Fig 5 Separate weights of the cortex and medulla of the thymus Symbols as in Fig 1

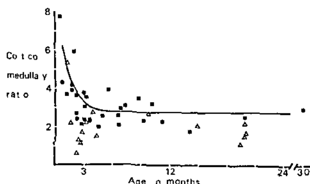


Fig 6 The ratio of cortical areas to medullary areas as estimated from planimetric measurements on one random section of the thymus Symbols as in Fig 1 The curve line refers to the combined SUD and ACC groups

relative size of cortex and medulla The amount of stroma is very small and was ignored in these estimates The results are shown in Fig 5 Both cortex and medulla increase rapidly in weight during the first three to four months of life The medulla is very small in the youngest cases, and the relative growth rate is much greater in the medulla than in the cortex Weight reduction with disease appears mostly in the cortex The tendency toward larger thymus weights in the ACC group than in the SUD group is reflected in both cortical and medullary weights

The ratio of the volume of the cortex to the volume of the medulla as estimated from

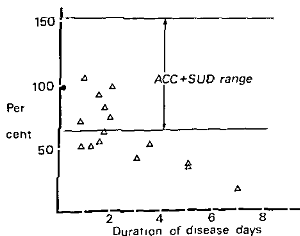


Fig 7 The ratio of cortical areas to medullary areas, expressed as per cent of the SUD + ACC curve in Fig 6, and related to the duration of illness Symbols as in Fig 3

planimetric measurements is shown in Fig 6 There is no systematic difference between the ACC and SUD groups, and a normal curve has been drawn on the basis of the two groups combined The cortico-medullary ratio decreases rapidly during the first three months of life For the rest of the period studied, the cortico-medullary ratio shows no obvious change

The relation between the duration of disease and cortico medullary ratio is shown in Fig 7 After two days of disease or less, most cortico medullary ratios are within normal range When disease has lasted for more than two days, the cortico medullary ratio is below normal range in all cases

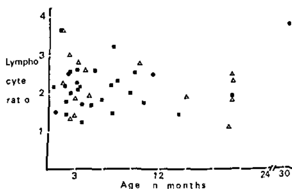


Fig 8 The ratio of the number of lymphocytes small and medium sized per unit section area in the cortex to the number of lymphocytes per unit section area in the medulla Symbols as in Fig 1

The ratio of the number of lymphocytes per unit section area in the cortex to the number of lymphocytes per unit section area in the medulla showed no consistent variation either with age or between the diagnostic groups, as shown in Fig 8

DISCUSSION

There is good agreement between the thymus weights of suddenly dead infants reported here and the weights given for similar groups of infants by *Hammar* (9, 10) and *Jacobsen & Voigt* (13) The infants which in the tables of *Boyd* (5) are recorded as 'found dead' or 'sudden death" had thymus weights that

average about 90 per cent of the present normal curve However, the individual variation is substantially larger in *Boyd's* cases In particular, there is an undue proportion of thymus weights below the range seen in *Hammar's*, *Jacobsen & Voigt's*, and the present material, which may indicate either that some of the infants in *Boyd's* material had been ill for some time prior to their apparently sudden death or that the general state of health and nutrition was more variable in her material Much lower values for average thymus weights have been given by authors who have not restricted their material to cases of sudden death (19 23)

The relative thymus weight is largests at about three months of age A similar maximum occurs in mice at two to three weeks of age both in germfree and in conventional animals (2, 26)

The relation of thymus weight to the duration of disease in the ILL group shows a large individual variation Much of this scatter is obviously due to individual variations that were present prior to disease Also differences in the strength and kind of stress in different cases may have been of some importance, since the degree of thymic involution is known to be dose dependent in animal experiments (12 21) Moreover the duration of disease may have been underestimated in some cases, since even fatal diseases like myocarditis and severe bronchopneumonia in infants may pass unnoticed by the surroundings until the baby is found dead in bed (3 4 16)

The relative size of cortical and medullary areas in histological sections showed less individual scatter than the weight parameters, particularly in the relation to the duration of disease in the ILL group Otherwise the data concerning thymus weight largely agree with those concerning cortico-medullary ratio in that individual values may or may not be within normal range after one to two days of disease and are below normal range after more than two days of disease It appears that little or no significant deviation from normal can be expected when a disease has lasted for less than 8 12 hours

The mechanisms of thymus weight reduction in acute involution are a halt in the mitotic production of new lymphocytes in the cortex and an accelerated disintegration of those present (6, 7, 8, 12, 17, 25) In a series of experiments including a single dosage of corticosteroids, bacterial toxins, or urethan thymus weights of laboratory animals have been found to be normal after about six hours and the first significant weight reduction has been noted at 12–18 hours after the treatment (7, 15, 18 25)

There is some evidence, epidemiological (22), bacteriological (14), as well as pathological (16, 20), that many cases of SUD may be caused by fulminant infections. The present observations concerning the time relations of acute thymic involution in acute illness, and the comparison with data from experimental work on animals, indicate that normal thymus weight and cortico-medullary ratio in SUD are compatible with death from fulminant infections of less than 8–12 hours' duration However these observations are of course equally well compatible with any of the other acute mechanisms that have been proposed as the explanation of SUD (24)

In the present material, thymus weight was slightly lower in the SUD group than in the ACC group This may possibly be a reflection of an imminent thymus weight reduction in response to disease in the SUD group, and it may not be fully justified to include SUD cases in the estimates of normal thymus weight However, the difference is small, and the number of cases is small It would be desirable to have this point retested in other studies

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HISTOLOGICAL DEMONSTRATION OF A BIPHASIC ACUTE INFLAMMATORY RESPONSE TO SUBCUTANEOUS TRANSPLANTS OF EHRLICH'S CARCINOMA

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Following treatment with phenylbutazone the acute inflammatory response to subcutaneous transplants of Ehrlich carcinoma cells was shown to be biphasic It is suggested that both phases of the inflammatory response may be a sequel to immunological lysis of the tumour cells by humoral antibody and complement

It is well known that an acute inflammatory response occurs around subcutaneous transplants of many mouse and rat tumours (see *Vasiliev* 1958) The biochemical changes in the zone of interaction between host and tumour tissues that is the zone primarily affected by this inflammatory response, have also been studied in detail (*Sylven* 1967) Recent experience at this Institute suggests that the inflammatory response per se may determine the mode of growth of subcutaneous transplants of the Ehrlich carcinoma, the acute inflammatory response favouring invasive rather than expansive growth (*Hartveit* 1969)

The timing of this acute inflammatory response has received little attention previously although its importance following intraperitoneal transplantation of these tumour cells was stressed some years ago (*Hartveit* 1965 a) The following experiment was set up to investigate the time relationship between transplantation of the tumour cells in subcutaneous tissues and the development of acute inflammation around them

In our untreated mice an acute inflammatory response is usually present around such tumour transplants over most of the growth period Variation in the intensity of this reaction is difficult to assess by conventional methods and previous attempts using only untreated mice failed to show any systematic differences However, in mice in which the inflammatory response was depressed by treatment with phenylbutazone a biphasic pattern emerged This was investigated as follows

MATERIAL AND METHODS

Mice of our closed colony (*Hartveit* 1961) were used at an age of 3½–4 months (weight ± S D,

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approximately the same pattern with an acute inflammatory response immediately following transplantation and again on day 14

Details of the Histology

In *untreated mice* the acute inflammatory response was present throughout most of the period investigated and was accompanied by infiltrative growth of the tumour cells

In the early stages of tumour growth (up to day 5) no sex difference was apparent An acute inflammatory response was present above the panniculus carnosus and below it between the muscle layer and the surface of the tumour transplant (Fig 2) The transplant itself showed central necrosis from day 2 Above the panniculus carnosus margination of granulocytes in the dilated blood vessels was marked (Fig 3) Below the panniculus tumour cells spread irregularly from the surface of the transplant into the inflammatory exudate (Fig 4) Infiltrative tumour growth became established in both sexes, the tumour cells growing through the panniculus At 5 and 7 days in the females the acute inflammatory response was less intense, but growth remained of infiltrative type On its return at 10 days the cytology of the inflammatory response did not differ from that seen in the early stages of tumour growth In the males infiltrative growth continued up to the end of the period investigated (Fig 5) The figure shows the advancing edge of the tumour, which has invaded the subcutaneous tissues above the panniculus This growth was accompanied by an acute inflammatory response Macrophages were present but few lymphocytes In contrast in the females the character of the tumour growth changed After day 15 the acute inflammatory reaction became less marked By day 21 the edge of the transplant was well demarcated from the surrounding connective tissue which contained few inflammatory cells (Fig 6), and the tumour cells formed layers parallel to the surface of the transplant

In the *treated mice* the histological changes were essentially similar in both sexes, although, as seen from Fig 1, the duration of the responses differed

The early acute inflammatory response was similar to that seen in the untreated mice (Fig 7), with oedema, granulocyte infiltration and necrosis of the tumour cells in the centre of the transplant This reaction, in particular the oedema between the panniculus carnosus and the transplant, resolved between days 3 and 13 (Fig 8), leaving some few granulocytes and macrophages in the tissues above the panniculus carnosus A band of connective tissue formed in the area of host tumour interaction below the panniculus In it the fibroblasts ran parallel to the surface of the transplant forming a line of demarcation between the tumour cells and the original host tissues (Fig 9) From day 5–18 in the females and day 9–21 in the males this band was infiltrated by lymphocytes and macrophages The microscopic structure of this zone has been described in detail previously at 9 days (*Hartveit & Halleraker* 1970) However, it was found that at 14 days in both sexes an acute inflammatory response returned (Fig 9) The capillaries in and above the connective tissue band became dilated (Fig 10) and granulocytes mingled with the lymphocytes and macrophages in this zone Oedema formed again, splitting the band of connective tissue from the panniculus carnosus (Fig 11) New capillaries formed in this area running at right angles to the surface of the transplant The tumour cells forming the surface of the transplant were at this time morphologically intact but by 16 days extensive tumour cell necrosis had occurred Only a few swollen tumour cells, adjacent to the connective tissue band remained intact (Fig 12) The acute inflammatory response continued (Fig 13)

By 21 days large areas of the transplant were replaced by new granulation tissue in which the capillaries ran at right angles to the surface of the original transplant (Fig 14) A few scattered islands of surviving tumour cells were found in all cases

males 29 2 ± 2 2 grams females 26 2 ± 1 4 grams)

Two groups were set up There were 34 males and 36 females in the treated group 12 males and 12 females in the untreated group

Phenylbuta one (1 mg dissolved in 0 1 ml triethyleneglycol) was given in one daily intra peritoneal dose to the mice in the treated group The untreated group received a needle puncture but no injection These controls are reported in stead of conventional controls receiving an injec tion of the solvent although it has been shown that the solvent does not effect the acute inflamma tory response (*Hartveit* & *Halleraker* 1970) A group of animals was required in which there was no chance that the mode of reaction had been altered by treatment The action of phenylbuta zone versus that of the solvent was not under in vestigation On the other hand a needle puncture was considered necessary to ensure that the han dling the animals received was comparable as the subcutaneous transplants were on the back and a certain amount of trauma could be expected when they were picked up for injection Treatment was started the day before tumour transplantation

The tumour was the Ehrlich carcinoma kept by serial intraperitoneal transplantation in our mice Each of the mice in both groups was given 0 05 ml of *whole tumour ascites from a 10 day transplant* subcutaneously on the back (6 × 10 tumour cells)

Experimental Procedure

At intervals of 2 or 3 days after tumour trans plantation 2 or 3 males and 2 or 3 females in the treated group and 1 male and 1 female in the un treated group were killed (see Fig 1) The skin of the back with the subcutaneous tumour was removed and fixed in 4 per cent buffered formalin Blocks of tissue were removed through the greate t diameter of the tumour at the point of greatest depth Sections of this paraffin embedded material were stained with haematoxylin and eosin

Histological specimens produced in this way have been described in detail previously (*Hartveit* & *Halleraker* 1970) The tumour cells lie as a rule mainly under the panniculus carnosus In un treated mice the blood vessels in the subcutis above the panniculus carnosus are usually involved in the inflammatory *reaction So too are the origi* nal vessels below the muscle layer and the newly formed vessels in the granulation tissue in the invasive *zone* Vessels in these 3 sites were studied in the present work

The histological *criteria* used to assess the degree *of acute* inflammatory reaction were *dilatation* of the small vessels with *margination* of granulo cytes the presence of *oedema* in the perivascular tissues and *granulocyte infiltration*

RESULTS

Some of the mice in both groups died as a result of *treatment and/or tumour growth* before the end of the experiment and a few of the early histological specimens proved unsatisfactory (*i e* tumour cells not found) The final material therefore consisted of 27 males and 25 females *in the treated group* 10 males and 11 females in the untreated group

Acute inflammatory response From Fig 1 it can be seen that the acute inflammatory response as judged by the criteria listed above, was present around the tumour trans plant in all the male *untreated control mice* throughout the period investigated In the females there was some indication that the response was less at 5 and 7 days returned at 10 days and then faded out from 17 days on Following *treatment with phenylbuta zone* this biphasic pattern became clearer in the females and appeared also in the males As Fig 1 shows the response in the treated males was intense up to day 4 but had faded out by day 11 It returned to full intensity *from day 14 to 18* but became slightly less intense later The treated females followed

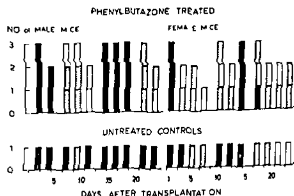
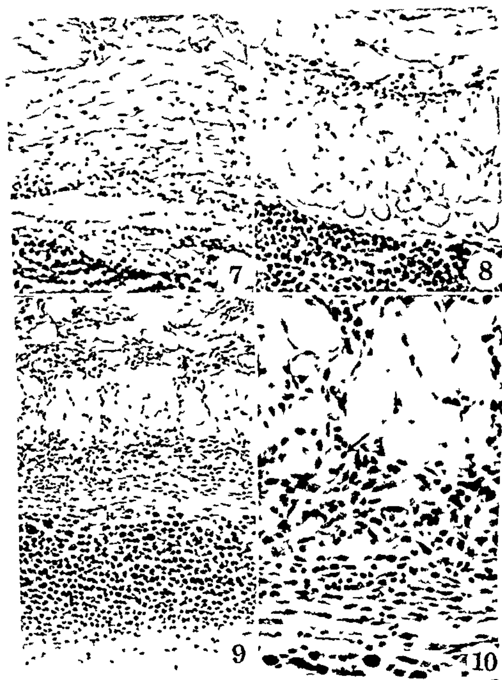


Fig 1 The acute inflammatory response around subcutaneous transplants of Ehrlich carcinoma cells related to time after transplantation in male and female mice treated with phenylbutazone and in untreated controls *For details of criteria of in* flammation see text

Black	columns 4	criteria fulfilled
Dark grey	2 3	
Light	1	criterion
White	0	criteria



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DISCUSSION

Treatment with phenylbutazone was used in the present work in an attempt to cut down the acute inflammatory response that normally occurs around subcutaneous transplants of the Ehrlich carcinoma in our mice. This object was achieved at some stages of tumour growth. However, the treatment used did not suppress the acute response directly following transplantation or the response 14 days after transplantation. These findings suggest that the acute inflammatory response to the Ehrlich carcinoma may be biphasic.

Other effects of phenylbutazone on tumour growth have been described in detail previously at 9 days (*Hartveit & Halleraker* 1970). The present study confirms the previous finding that treatment with phenylbutazone alters the growth pattern of Ehrlich's carcinoma in our mice. In untreated mice growth is of infiltrative character until the later stages. (These later stages, day 15 on, are under further investigation and will be discussed in detail in a subsequent paper.) On treatment tumour growth becomes expansive and a band of lymphocyte and macrophage infiltrated connective tissue forms around the transplant.

This round cell response preceeds the second peak of acute inflammation. In untreated mice lymphocyte infiltration is minimal. In the absence of such cells the acute inflammatory reaction with subsequent organisation that is characteristic of tumour growth in untreated mice appears to facilitate tumour cell infiltration, providing the proliferating tumour cells with a highly

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Fig 2. Subcutaneous transplant of Ehrlich carcinoma cells in *untreated mouse* (Day 4 male). Note acute inflammatory response with oedema and leukocyte infiltration above and below the panniculus carnosus. Surface of tumour transplant at bottom of picture. (H & E × 150)

Fig 3. As Fig 2.
Note margination of granulocytes in dilated vessel above panniculus carnosus. (H & E × 370)

Fig 4. As Fig 2.
Note irregular distribution of tumour cells (↑) in inflammatory exudate. Surface of transplant at bottom of picture. (H & E × 370)

Fig 5. As Fig 2 (Day 21 male).
Note infiltrative tumour growth above and below panniculus carnosus accompanied by inflammatory response. (H & E × 150)

Fig 6. As Fig 2 (*Day 21 female*). Contrast Fig 5.
Note sharp demarcation between tumour cells forming layers on surface of transplant and surrounding connective tissue containing few inflammatory cells. (H & E × 150)

Fig 7. Subcutaneous transplant of Ehrlich carcinoma cells in mouse *treated with phenylbutazone* (Day 2 female).
Note oedema between tumour transplant (bottom) and panniculus carnosus (top). (H & E × 150)

Fig 8. As Fig 7 (Day 3 female). Contrast Fig 7.
Note lack of oedema between transplant (bottom) and panniculus carnosus (centre). (H & E × 150)

Fig 9. As Fig 7, treated mouse (Day 14, male).
Note band of connective tissue in centre of picture between panniculus carnosus and surface of transplant. (H & E × 150)

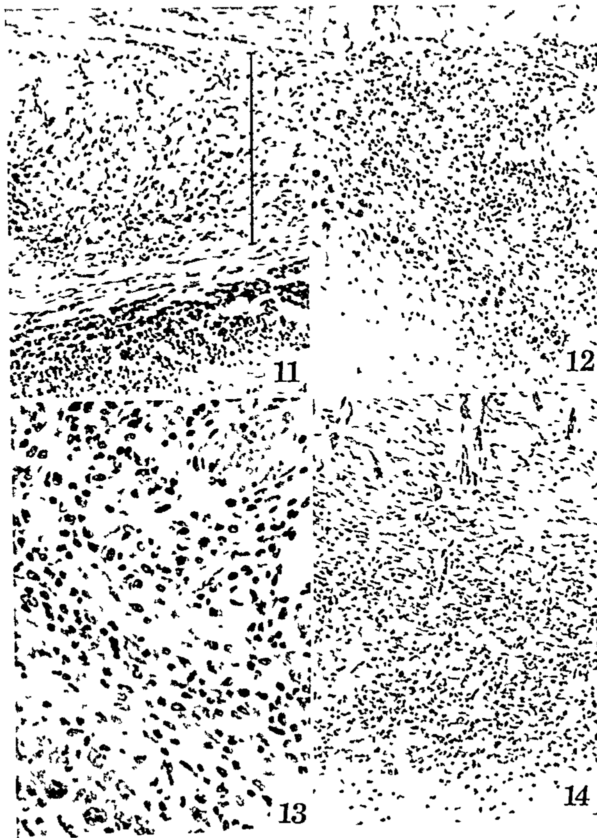
Fig 10. Detail of Fig 9 treated mouse.
Note dilated capillaries in and above (↓) connective tissue band. (H & E × 370)

Fig 11. As Fig 7 treated mouse (Day 14, male).
Note inflammatory exudate between panniculus carnosus and connective tissue band (I) above tumour transplant and morphologically intact tumour cells at upper edge of transplant. (H & E × 150)

Fig 12. As Fig 7 (Day 16 male).
Note extensive tumour cell necrosis (bottom left) and wide connective tissue band between edge of transplant with few remaining tumour cells, and panniculus carnosus. (H & E × 150)

Fig 13. Detail of Fig 12.
Note swollen tumour cells (bottom left), dilated vessels and granulocytes mixed *with* lymphocytes and macrophages in the connective tissue band. (H & E × 150)

Fig 14. As Fig 7 (Day 21 male).
Note capillaries in new granulation tissue run at right angles to surface of necrotic transplant (bottom). (H & E × 150)


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antibody effect both the above actions would be demonstrated In the presence of the small amount of humoral antibody carried over from the donor on the surface of the tumour cells immunological lysis of some of the tumour cells would occur The remainder profit from the resultant acute inflammatory response, and infiltrative tumour growth becomes established This would be a direct parallel to the situation on intraperitoneal transplantation (*Hartveit* 1965b) Later the host will produce humoral antibody This could account for the second acute inflammatory response seen in the present experiment But this time the majority of the tumour cells are killed Few remain to profit from the resultant inflammatory response

With intraperitoneal transplants the presence of anticomplementary inflammatory exudate enables the antibody coated tumour cells to survive in the presence of complement (*Hartveit* 1965b) It is tempting to suggest that a similar mechanism may operate with subcutaneous transplants In untreated mice the exudate produced at the growing edge of the tumour, in response to lysis of some of the tumour cells, enables a rim of tumour tissue to survive In the treated mice this delicate balance is upset and the relative lack of exudate may permit lysis in this rim to

To return to the lymphocyte response, and consider it on the basis of the above hypothesis We have to explain why a lymphocyte response should occur in treated mice but not in untreated mice Phenylbutazone will, by reducing capillary permeability (*Wilhelmi* 1950), make it more difficult for antibody globulin to reach the tumour cell surface than will be the case in untreated animals This would give more time for information as to the tumour cell surface to reach the reticuloendothelial system, in other words the afferent mechanism of enhancement due to humoral antibody would fail In due course a lymphocyte response would result, as in the present experiment However, by this time the tumour cell surface would be antibody coated The efferent mechanism of enhancement may then come into play, preventing the lymphocytes from homing effectively on their target

On the basis of the above considerations it is therefore suggested that humoral antibody may be responsible for both phases of the acute inflammatory response seen in the present experiment The difference in outcome, *i e* facilitation of tumour growth in the first phase and tumour cell necrosis in the second, is more likely to be due to an effect of dosage than to a difference in the underlying mechanism involved

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vascular stroma On the other hand the second peak of inflammation seen in the treated mice that occurred in the presence of lymphocyte infiltration, did not facilitate tumour growth Although a highly vascular connective tissue stroma was produced, massive tumour cell necrosis occurred This granulation tissue did, however, contain small areas with surviving tumour cells Survival time studies on groups of mice similar to those used in the present experiment have failed to show any statistically significant difference between treated and untreated groups, although tumour size was significantly reduced in treated females (0 001 > P) Whether these mice died of, or with their tumours remains an open question, as is the effect of long term treatment with phenylbutazone on survival time in these mice

The present histological findings thus suggest that the acute inflammatory responses seen in this study may be of 2 types, *i e* that occurring in the absence of lymphocyte infiltration, and that occurring in its presence These two types have been shown here to differ in outcome This raises the question as to whether the underlying stimulus that caused these two responses was different *Sparck* (1962) has also suggested that the inflammatory response to a tumour transplant may be of two types A non antigenic transplant is said to elicit a non specific response with resultant stroma formation, while an antigenic transplant elicits an immunologically specific response with subsequent tumour cell death This thesis cannot be upheld in the present case as antigenic tumour cells were involved in both reactions

Ehrlich cells, such as those used for transplantation in the present experiment are known to be coated with immunoglobulin (*Thunold* 1968) derived from their previous host Such cells are also known to undergo immunological lysis if complement is available on int

to call forth

veit 1965b)

first peak of inflammation seen in the mice in all groups in the present experiment may be due to the products complement mediated immunological lysis This would imply that the central necrosis seen in untreated transplants, which has been recorded previously (*Hartveit* 1969 (Fig 6)), may not be due solely to lack of nutrients This is under further investigation

The second peak of the acute inflammatory response seen in the treated mice was proceded by lymphocyte infiltration This could indicate that the lymphocyte was the cytotoxic agent responsible for the extensive tumour cell necrosis that followed This would be in keeping with current views on the rôle of the lymphocyte in tumour immunity (*Alexander & Fairley* 1967) But on the other hand the tumour cell necrosis in the present experiment spread from the centre of the transplant out to the periphery where the majority of the lymphocytes were to be found The cytotoxicity of the lymphocytes in the present case is thus in doubt

Even so the timing of the second peak suggests that an immunological response may be involved, and once again the possibility of immunological lysis due to complement binding humoral antibody has to be considered It is commonly held that humoral antibody increases tumour growth and its inherent cytotoxic properties are negligsed (*Alexander & Fairley* 1967) The action of humoral antibody as 'enhancing antibody' has been widely publicised Enhancing antibody is said to cover the target cell and so prevent further information on its antigenicity reaching the reticuloendothelial system (afferent mechanism), and to prevent already primed lymphocytes from reaching their target (efferent mechanism) Both these mechanisms have been convincingly demonstrated under certain experimental conditions (*Möller* 1963) But this is not the whole story Under other experimental conditions the cytotoxicity of humoral antibody *in vivo* has also been demonstrated (*Kaliss* 1958) These two opposing actions can be dose dependent (*Gorer & Kaliss* 1959)

If the findings in the present experiment are to be explained on the basis of humoral

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RUPTURE OF THE RIGHT VENTRICLE OF THE HEART IN A CASE OF ADVANCED HEART AMYLOIDOSIS

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A case of advanced heart amyloidosis with rupture of the right ventricle is reported

Rupture of the wall of the right ventricle of the heart is rare, but is sometimes met with as a complication of heart infarction Cases of spontaneous rupture not in association with an infarct have also been reported (*Pulvertaft* 1932, *Howell* & *Piggot* 1950, *Knight* 1962) Common to all of these is that neither a post mortem nor a microscopical examination revealed morbid changes in the ruptured area of the heart It seems therefore of value to describe a case of advanced heart amyloidosis with rupture of the wall of the right ventricle

CASE REPORT

A seventy-year-old, obese watchman, with known auricular fibrillation, became engaged in fisticuffs with a drunkard However, it is impossible to say whether he was hit or not At a rather early stage of the brawl he collapsed against a wall and showed no signs of life when help arrived

The post mortem revealed a heart greatly enlarged in relation to body weight (heart weight 750 gm body weight about 95 kg, body length 174 cm The right ventricle was dilated and the wall measured at the base about 3 mm The wall of the left ventricle measured at the corresponding place about 25 mm and the apex of the heart was formed by the left half of the heart Valves and openings were normal, apart from a slight calcification of the most basal parts of the aortic valves The coronary arteries left the root of the aorta in a normal way They showed a moderate degree of arteriosclerosis The arteriosclerosis was most pronounced in the circumflex and in the descending branch of the left coronary artery In places the lumens were markedly constricted, but no fresh thrombo[illegible]
total lume[illegible]
ously stiff [illegible]
to pale br[illegible]
evidence of fresh or old infarcts was seen

In the front wall of the right ventricle was a rupture, about 15 mm long, running parallel to and about one centimeter from the septum The edges were frayed and there was a number of small though not pronounced bleedings in the muscles adjoining the rupture The pericardium contained more than 200 cc blood and blood clots When the chest was opened, the blood could be discerned through the pericardium, which was completely intact At the post mortem there were no signs of external violence

The microscopical examination of sections from the ruptured area as well as from the wall of the left ventricle did not reveal fresh infarction, myocarditis or marked infiltration of fat

A number of sections from different parts of the heart were studied by fluorescent microscopy The sections were stained with thioflavine-T to reveal amyloid (*Kurban* 1960, *Hobbs* & *Morgan* 1963) Practically all the heartmuscle fibres fluoresced intensively greenish yellow (Fig 1) The sections had the same intensive fluorescence as a control section from a known case of amyloid disease stained at the same time, while sections from a

Received 17 vi 70

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FLUORODEOXYURIDINE-INDUCED MALFORMATIONS IN MICE

Studies on the Early Stages of Teratogenesis

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At different stages of gestation, 104 mice were treated with fluorodeoxyuridine (FUDR) 34 pregnant mice served as controls The experimental mice were examined at varying intervals after the FUDR injection At term, the young exhibited the malformations of the limbs and skull so well-known after administration of FUDR In addition, there was an increased number of resorptions 24 hours after the injection on the 9th–12th day of gestation, vascular dilatations and fresh haemorrhages were found at the sites of predilection of malformations Foetuses removed 2 or more days after the injections showed characteristic saccular haematomas in the limbs and tail as well as flat, widespread haematomas in the skull At increasing intervals after the injections, the haematomas decreased in number and were rarely present in foetuses at term As the number of haematomas decreased the number of malformations increased Daily laparotomies, from the 12th to the 18th day on 5 mice treated on the 11th day with FUDR revealed extremity haematomas which disappeared on the 13th–18th day Electron microscopic pilot studies of 2 foetal limbs showed 24 hours after the injection of FUDR, red blood cells in an extra vascular situation The control mice had 354 normal foetuses and 8 per cent spontaneous resorptions This study, which was a screening experiment, showed that FUDR induces vascular injury with haemorrhages and formation of haematomas, resulting in congenital malformations The vascular injuries are interpreted as the primary action of FUDR on the cellular level

Experimental teratology has so far been concentrated mainly on "model experiments", inducing under given conditions, malformations similar to those known from clinical experience

However owing to species differences, it has proved impossible to draw direct conclusions from these experiments to the causal relations in human malformations

Accordingly the understanding of the numerous problems concerning the abnormal developmental processes can hardly be extended by such model experiments or by recording malformations as they manifest themselves in foetuses at term This is more likely to be accomplished by studying the basic morphological, biochemical, and cytological changes which take place during the development of the malformations, i e by studying the embryo- and organogenesis

The preliminary results to be reported below were obtained in an experimental study of the morphological changes in the early stages of the abnormal development after

Received 19 xi 70



Fig 1 Section from the ruptured heart stained with thioflavine T Almost all of the heart muscle fibres show an intensively greenish yellow fluorescence (white in the picture) Magnification about 125 × in the microscope

young healthy heart did not fluorescence when stained with thioflavine T

In paraffin sections from the ruptured area stained with congo red a great number of the heart muscle fibres showed a red colour When the sections were examined in polarized light a green colour was observed This green colour is said to be specific for amyloid (*Missmahl & Hartwig* 1953) On the other hand congo red did not stain the muscle fibres red in sections from a healthy heart nor was a green colour observed when the sections were examined in polarized light Gentian violet stained the majority of the heart muscle fibres violet blue to reddish

Lungs lymph nodes liver spleen kidneys and thyroid gland were microscopically normal apart from a slight mainly large globular fatty deposit in the liver and deposits of amyloid in the walls of the splenic arteries

No case of heart amyloidosis complicated by rupture of the heart is to the best of my knowledge reported in the literature In this case, it seems likely that the amyloid seriously impaired the ability of the heart wall to resist a sudden intraventricular rise in pressure, due to a psychic strain, with a rupture of the relatively thin wall of the right ventricle as a consequence Some support for this is the volume of blood (ca 200 cc) found in the pericardium The rupture of the ventricle wall, due to a heart infarct always causes rather massive bleedings into the pericardium (about 200–500 cc), and a similar volume of blood is to be expected in rupture of the right ventricle arising *intra vitam*

In this case the chest could have been hit by a clenched fist but this is not enough to cause rupture of the heart In order to cause a rupture the assault against the chest wall must be rather severe, as for example in a car accident At the autopsy, however, no signs of external violence were found (abrasions bruises) Therefore the rupture could not have been due to external violence against the chest

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Fig 2 Total spina bifida in an 18-day-old mouse foetus removed from a pregnant mouse injected 8½ days previously with 2 mg FUDR



Fig 3 A 12-day-old mouse foetus removed from a pregnant mouse injected 2½ days previously with 2 mg FUDR Note haematoma in the theca cranii and at the mandible



Fig 4 Haematomas in both hind legs of a 17 day old mouse foetus removed from a pregnant mouse injected 5¾ days previously with 2 mg FUDR Tail short and misshapen

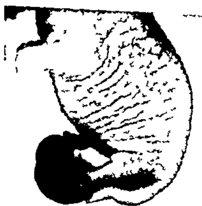


Fig 5 Haematoma in the left hind leg of a 17 day old mouse foetus removed from a pregnant mouse injected 5¾ days previously with 2 mg FUDR No outlining of digits on the forelegs and the right hind leg Tail deformed

exposure to a substance which is consistently teratogenic

MATERIAL AND METHODS

The experimental animals were mice of an inbred albino strain which has never exhibited spontaneous malformations

The time of copulation was fixed as the middle of two hours spent by the male in the same cage as the experimental female The finding of a vaginal "plug" was taken to indicate that the mouse was pregnant Foetal age was calculated from the time of copulation

On the basis of *Murphy's* (1960) studies we selected as teratogenic agent a fluoro-substituted thymidine analogue, 5 fluoro-2-deoxyuridine(FUDR), pharmacologically an antimetabolic cytotoxic drug

After preliminary studies, the dosage was fixed as 2 mg/mouse, *i e* approx 60 mg/kg The agent was dissolved in sterile water and injected as a single dose intraperitoneally at different stages of gestation As already mentioned, we devoted most interest to the early stages of teratogenesis and therefore killed the majority of the mice with ether at varying times after injection of the teratogenic agent A small number of mice were left until term around the 19th–20th day The foetuses were removed, fixed in formalin, weighed, and inspected for gross abnormalities and deformities In addition, we recorded the number of resorptions, *i e* the implantation sites in the uterus where a foetus had perished At the same time, and in the same way, we studied foetuses from control mice of the same copulation age as the foetuses of the experimental series In the control mice too, the number of resorptions were recorded

The material comprises 109 treated pregnant mice with a total of *1140 foetuses and resorptions* and 34 pregnant control mice with 384 foetuses and resorptions Five of the treated mice injected on the 11th day, were laparotomized on the 12th day and on the subsequent days until term

Electron microscopic pilot studies were performed on the limb primordia from two foetuses removed on the *12th day, 24 hours after the injection* of 2 mg of the teratogenic agent

RESULTS

In the preliminary investigations, a dosage of 2 mg of FUDR per pregnant mouse proved well suited for the experiments, *i e* this dose proved to result in a suitable number of reproducible malformations and resorptions 1 mg of FUDR/mouse was found to have no or little teratogenic effect, while 3 mg resulted in a foetal mortality close to 100 per cent

The 34 pregnant mice of the control series comprised a total of 354 grossly normal foetuses and 30 resorptions, corresponding to a primary litter size of 11 3, 8 per cent of which were in the course of spontaneous resorption *No gross deformities* were observed during the experimental period (When the experiment had been concluded we found after a period of greatly decreased conception rate in the stock, foetuses with cranial malformations (encephalocele) and tail defects of unknown cause Spontaneous malformations of the limbs were not observed)

The 109 treated pregnant mice had 675 foetuses—normal and malformed—as well as 465 resorptions, corresponding to an average primary litter size of 10 5 and a resorption rate of 41 per cent

The foetuses of the treated mice exhibited *general inhibition of growth* as compared with the control foetuses

Fig 1 gives the percentage distribution of normal, malformed, and resorbed foetuses in relation to the time of injection of the teratogenic agent (2 mg FUDR) in 104 treated litters The broken line indicates the spontaneous resorption rate in the control series It is apparent from the figure that the conception product was not influenced by the teratogenic action during the first days while

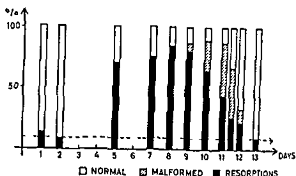


Fig 1 Percentage distribution of normal malformed, and resorbed mouse foetuses in relation to the time of injection of the teratogenic agent (2 mg fluorodeoxyuridine—FUDR) in 104 litters The broken line indicates the spontaneous resorption rate in the control group

strable malformations with or without haematomas at different times

In the 5 laparotomized mice 31 foetuses and 30 resorptions were recorded On the 13th day haematomas in the limbs of 24 foetuses were distinctly visible through the uterine wall During the subsequent days the number of haematomas decreased When the mice were killed on the 19th day there was only one small haematoma in a hind leg

Electron microscopic studies of the limb primordia from two 12 day old foetuses removed 24 hours after the injection of FUDR disclosed in specimens from the distal parts numerous extravascular erythrocytes The capillaries and other vessels could not be definitely assessed because as far as we know there is no elektron microscopic investigations concerning the normal vascular development in the limbs The more profound layers of the limb mesoderm exhibited cellular changes which perhaps might be interpreted as incipient degenerative changes The most superficial mesodermal layers of cells as well as the extodermal lining were normal

DISCUSSION

Being a screening study the present investigation suffers from certain systematic shortcomings However a preliminary report was felt to be justified as the named haematomas do not appear to have been reported previously in connection with drug induced malformations

The size of the dosage the nature and localization of the malformations as well as the time of maximum sensitivity to FUDR correspond accurately to the findings of *Murphy* (1960)

It seems an established fact that FUDR administered in a dose of 2 mg/mouse around the 10th 12th day of pregnancy will regularly induce malformations mostly in the limbs Embryologically this period corresponds to the stage in the mouse at which limb buds appear (about 10½ days after the conception) develop and differentiate until they present distinct interdigital grooves on the 13th day (*Otis* & *Brent* 1954)

It has not been finally elucidated whether FUDR has a particular affinity for the mesodermal tissue in the limb primordium or whether it is a question of a systemic cytotoxic action manifesting itself only in the most actively proliferating and therefore most vulnerable tissue at a given time cf chemotherapy of cancer (*Plattner* 1964) *Jurand* (1961) demonstrated that certain cytotoxicants of the group nitrogen mustard derivatives when administered to mice, exert a specific toxic action upon the neural tube and upon the mesoderm in the extremity primordia at the same time general inhibition of growth takes place In electron microscopic studies of the extremity primordia *Jurand* found necrotic changes in the deeper layers of the mesoderm while the 2 or 3 most superficial layers of mesodermal cells like the ectodermal lining proved normal In particular, the apical ectodermal cap which acts as an inductor in the development of the extremity was normal

The characteristic haemorrhages in and around the extremity primordia found by us have not been described in connection with the numerous recent studies of drug induced malformations A reasonable explanation is that in these previous studies interest has been taken only in the foetuses around term when the *haematomas* have disappeared (Fig 7), leaving 'scars in the form of finished malformations club foot syndactylia brachydactylia deformities of the skull and tail

The postulation that the pathogenesis was vascular lesions vascular injuries with haemorrhage formation of haematoma *necrosis* and arrest of growth absorption of the haematomas and development of malformations was confirmed by the investigation of the laparotomized mice in which the successive changes could be followed in the same foetuses *in utero* from the 12th day to term On comparison with normal foetuses (Fig 6) it seems that the injection of FUDR after the digits are outlined may induce the named vascular changes resulting not only in arrest of growth but in the destruction of

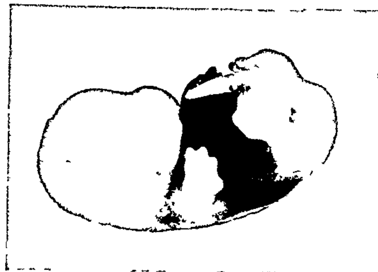


Fig 6 Normal mouse foetus aged 11 days 6 hours The outlining of digits is distinct, especially on the forelegs The age of this foetus corresponds to the time of injection of 2 mg FUDR in mice illustrated in Figs 4 and 5 The figure seems to show that the digital outlining has developed before the occurrence of haematomas, *i e* before the occurrence of malformations manifesting themselves as absence of digital outlining or of the foot

it is not in contact with the maternal circulation After nidation, which occurs in the mouse on the 4th day (*Otis* & *Brent* 1954) and during the subsequent 4 days or so, the primitive embryo reacts in accordance with an "all-or-nothing" law either all cells perish, the foetus dies and becomes resorbed, or else the development continues in the normal way, resulting in foetuses in which no macroscopic abnormalities are demonstrable With increasing differentiation of the tissues, on the 9th–11th day, a more selective action appears to take place, manifesting itself in an increasing number of surviving, but malformed foetuses After the 12th day the sensitivity decreased, and after the 13th day it was not possible to induce malformations by the dose employed

The typical malformations found in newborn young and foetuses removed at or shortly before term were cleft palate, brachygnathia, cranial deformities with or without meningocele and encephalocele, malformations of the limbs such as club-foot syndactylia and brachydactylia, as well as a short, angular tail In a few cases a whole limb was missing or rudimentary Vertebral malformations, in the form of spina bifida were also seen (Fig 2)

Foetuses removed 24 hours after the injection of FUDR showed none of the typical malformations On the other hand, they exhibited greatly dilated vessels in the tail, limbs, and skull, often with fresh haemorrhages or fluid-filled bullae Two or three days after the injections, flat, extensive haematomas were found in the skulls of 11–12-day-old foetuses (Fig 3), and saccular haematomas (Figs 4–5) at the sites of predilection of malformations were found in the limbs and tail of 13–14-day-old foetuses (By way of comparison Fig 6 shows a normal foetus removed at the time when FUDR was injected into the mice carrying the foetuses shown in Figs 4 and 5) At increasing intervals after the injections the haematomas decreased in number In foetuses at term they were rarely seen, but at the same time actual malformations increased in frequency Fig 7 illustrates the number of haematomas in per cent of the total number of demon

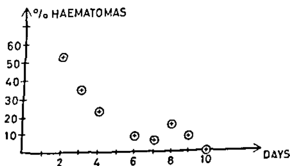


Fig 7 Percentage of demonstrable malformations in the form of haematomas related to the interval between injection of FUDR and removal of the foetuses

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ACCELERATION OF CASEIN INDUCED AMYLOIDOSIS IN MICE BY IMMUNOSUPPRESSIVE AGENTS

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The effect of various immunosuppressive agents on the experimental mice amyloidosis has been examined The mice were pretreated with 21 injections casein, on day 1, 3 and 5 after this treatment 12 immunosuppressive agents were applied to 12 groups of mice in dosis corresponding to LD_{10} All drugs applied accelerated the amyloidosis, even they differ in metabolic blocking potencies The results seem to indicate that any interference with the immune apparatus of the mice favours the development of amyloidosis

Teilum has shown (1952) that, in mice treated for several weeks with injections of casein, a dose in itself insufficient to cause amyloidosis, injections of cortisone for four to five days promptly resulted in the appearance of amyloid Later *Teilum* (1954) showed that nitrogen mustard had the same effect on experimental amyloidosis The same results were achieved by *Christensen* & *Hjort* (1959) using X-ray as accelerating agent

The above mentioned experiments led *Teilum* (1964) to the "two-phase cellular theory' 1) a pyroninophilic phase, due to a heavy antigen stimulation 2) an amyloid phase as a result of an "exhaustion" of the immune apparatus

The present study was performed in order to examine whether other immunosuppressive agents were able to accelerate casein induced amyloidosis in mice, in spite of their wide variations in their mode of action on the synthesis of D N A , R N A , enzymes etc in the cells

Received 28 v 70

MATERIAL AND METHODS

The animals were randomly selected mice from our inbred colony of the C3H strain Sex distribution was equal, all mice were between 8 and 10 weeks of age at the beginning of the experiment, weighing from 23 to 27 Gm They were fed on oats

97 mice were given a total of 21 daily injections of ½ ml of a 5 per cent solution of sodium caseinate subcutaneously The mice were separated into 13 groups and treated as shown in *Table 1*

The day after the last injection of the above mentioned agents all recipients were killed The spleen and the liver were fixed in neutral formalin and embedded in paraffin Sections were cut 5 microns thick and stained with haematoxylin-eosin, methyl green pyronine, alkaline Congo red, and the P A S stain Amyloid was identified by its morphology and by its birefringence with Congo red under crossed polars

The degree of amyloidosis, if any, was evaluated on sections of spleens and livers according to the semiquantitative method, ranging from 0-6, as described by *Christensen* & *Hjort* (1959)

RESULTS

The results are outlined in Table 2

In the control group consisting of seven mice, no amyloid formation was found; these

the outlined digits and other extremity structures

As early as in 1927, *Bagg published an* extremely detailed description of similar changes during the development of hereditary *malformations in mice In the offspring of* X-ray irradiated female mice he recorded, in the 3rd to 19th generation, a large number of malformations—in most cases combinations of renal aplasia and extremity defects, such as club-foot, syndactylia and polydactylia The earliest changes were found by *Bagg* on the 12th–13th day in the form of a subepithelial effusion of a clear, lymph-like fluid, a 'bleb" comprising a major or minor part of the extremity primordium The next stage was haemorrhage in these '*blebs*' which transformed them into characteristic saccular haematomas The haematomas were *most often found on the 14th–15th day and* thereafter appeared to be absorbed leaving typical foot deformities In a few newborn young the malformed feet showed remnants of haemorrhage which however, disappeared completely in the course of the first days *post partum Bagg* too could follow the successive changes by laparotomy

In hyperthermia experiments on chick foetuses *Öhre Nielsen* (1967) found the same changes which we observed after injection of FUDR

CONCLUSION

The morphological changes to occur during the development of extremity malformations in mice may be observed whether the underlying cause is genetic (endogenous) or exogenous, due to a toxic action in time relation to the development of the limbs It is reasonable to assume that in *Bagg s* experiments (1927) using X ray irradiation of female mice as well as in the present experiments using injection of FUDR it has been *a question of basic changes in the nucleic* acid structure of the cell nuclei In view of the consistent findings of haemorrhage and haematoma formation, resulting in malformations, it is reasonable to presume that injury to the vascular endothelium is the primary change on the cellular level

By continued investigations it will be endeavoured to elucidate the problem concerning the apparently specific action upon the vessels (mesoderm) and to ascertain whether this action occurs also with teratogenic agents of a specific biochemical nature different from FUDR

The action upon the vessels observed by us following injection of FUDR has so far been reported only in generation experiments *following X ray irradiation* of female mice and in non specific experiments using hyperthermia

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TABLE 2 *Effect of Immunosuppressive Treatment on Experimental Amyloidosis*

Group no	Number of mice	Treatment	Mortality	Amyloid incidence	Mean degree of amyloid in spleen	in liver
1	7	Casein	none	0/7	0 (0–0)	0 (0–0)
2	8	Cortisone	none	2/8	1 (0–3)	+ (0—+)
3	7	Chloramph	none	7/7	4 (4–5)	+ + (0— + + +)
4	7	Erasol	none	7/7	4 (3–5)	+ (0— + +)
5	8	Busulphan	1	7/7	4 (3–5)	+ (0—+ +)
6	8	Natulan	none	8/8	4 (3–5)	+ + (0—+ + +)
7	7	Methotrexate	2	3/5	2 (0–5)	0 (0—+ + +)
8	8	6 mercaptopurine	2	6/6	4 (2–5)	+ + (0—+ + +)
9	7	5-fluoruracil	2	5/5	4 (1–5)	+ + (0—+ + +)
10	8	Vinblastine	none	8/8	4 (2–5)	+ + (0—+ + +)
11	7	Actinomycin D	1	6/6	4 (3–5)	+ + (0—+ + +)
12	7	Daunomycin	1	6/6	5 (5–5)	+ + + (+ + +)
13	8	Imuran	1	7/7	5 (4–5)	+ + (+—+ + +)

with the genetic apparatus of these cells The direct metabolic death means a process which includes complete cessation of metabolic activity, visible necrosis and disintegration, and is seen especially in lymphoid tissue within a few hours after administration of corticosteroids, nitrogen mustard and actinomycin The so called *reproductive death*, is a damage leading to metabolically active cells, synthesizing protein and sometimes D N A and R N A The cells, however, being unable to carry out cellular replication This damage can be seen after application of radiation, nitrogen mustard, 6-mercaptopurine, folic acid antagonists and azathioprine (*Berenbaum* 1967)

The most conspicuous feature of the present results is the uniformity of the degree of amyloidosis between individual groups as compared to the negative controls not receiving cytostatic treatment From this observation it appears that obvious conclusions regarding the mechanism responsible for acceleration of amyloid formation in this experimental system cannot be drawn The immunosuppressive drugs employed represent a wide variety of metabolic blocking potencies However their sole common denominator in terms of function is apparently their capacity of interfering with immunocompetent cells Therefore it seems justified to ascribe their amyloid enhancing effect to an interference with the immune apparatus of the casein-treated animal

It has been extensively discussed whether an immune reaction was a mandatory step (*Janigan & Druet* 1968, *Janigan* 1969) in experimental amyloid formation Several workers have suggested a necessary immunological engagement in the primary phase of amyloidogenesis (*Teilum* 1964, *Ranløv* 1967, *Hardt & Ranløv* 1968). The present results seem to favour such a concept

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TABLE 1 *Immunosuppressive Treatment after 21 Injections of Casein*

Group no	Number of mice	Treatment (following 21 casein injections) on day 1, 3 and 5	Cellular functions blocked
1	7	0 5 ml 5 per cent Na caseinate i p	?
2	8	0 3 mg Cortisone i p	Inhibition of R N A synthesis
3	7	40 mg Chloramphenicol i p	Interference with m R N A attachment to ribosomes
4	7	0 05 mg Erasol (mechlorethamine) i p	Crosslinking of D N A
5	8	0 45 mg Busulfan (alkanesulfonic acid ester) i p	Rupture of D N A chains?
6	8	3 6 mg Natulan (methylhydrazine) i p	Rupture of D N A chains?
7	7	0 08 mg Methotrexate (methylaminopterium) i p	Prevents D N A synthesis blocking the reduction of folic acid to tetra hydrofolic acid
8	8	2 7 mg 6 mercaptopurine	Interference with the R N A synthesis
9	7	0 9 mg 5 fluoruracil i p	Blocks the D N A synthesis
10	8	0 015 mg Vinblastine i p	Produces metaphase arrest, mechanism unknown
11	7	0 002 mg Actinomycin D i p	Inhibits R N A synthesis by blocking D N A template
12	7	1 2 mg Daunomycin i p	Inhibits both D N A and R N A synthesis being attached to the two polynucleotide chains in D N A
13	8	25 mg Imuran (azathioprine) i p	Interference with the R N A synthesis

All doses were equivalent to I D_{10} dose (*Freireich et al* 1966)

mice were treated with a total of 24 injections of 5 per cent Na-caseinate

The experimental groups all showed a severe degree of amyloidosis in the spleen and to a lesser extent in the liver The distribution of amyloid followed the same pattern in all groups

DISCUSSION

The experiment showed that any immunosuppressive agent applied proved successful in accelerating the amyloid induction in the mice pretreated with casein

After 21 injections of sodium caseinate a high amount of pyroninophilic cells are present especially in the spleen Any interference with the functions of these cells might lead to degeneration of these cells and at the same time cause an increasing amount of P A S positive cells to appear, which on their side are known to be involved in amyloid formation (*Teilum* 1964)

The effects of the immunosuppressive agents are due to either direct killing of immunocompete

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LUNG PATHOLOGY IN RESPIRATORY DISTRESS FOLLOWING SHOCK IN THE ADULT

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Twenty cases of progressive respiratory distress (PRD) in adult patients following shock in connection with trauma, post-operative complications or serious acute diseases are reviewed. The development of pulmonary consolidation with the formation of hyaline membranes (HM) in these cases is described in detail. Organization of the HMs may result in fibrous obliteration of respiratory bronchioles and alveolar ducts, if survival is sufficiently long. This is a highly characteristic picture. Thin HMs without organization, interpreted as early lesions, were found among twelve additional cases treated by artificial ventilation; these patients did not develop PRD but died of other causes. The relation of the pathological findings to certain clinical factors such as length of survival with PRD, duration of mechanical ventilation and oxygen concentration, has been investigated. The results seem to concur with the theory that the pulmonary lesions are precipitated by inadequate tissue perfusion and possibly also intravascular coagulation during shock, leading to endothelial damage and reduced alveolar surfactant. Probably there are many important contributary factors, such as the mechanical effects of artificial ventilation, high oxygen concentration, infection, etc. 'The respiratory distress lung' seems to be an appropriate morphological term for this type of lung lesion, which may also be precipitated by uraemia and viral pneumonia, for example, and greatly resembles the hyaline membrane disease in the newborn.

Renal and hepatic parenchymatous degeneration and necrosis are the lesions most often encountered in patients who have died of shock of various aetiologies. Adrenal lesions are also common and sometimes myocardial and cerebral lesions may be found. In recent years, parallel with the rapid development of intensive care, severe pulmonary lesions have been observed with increasing frequency. After a free interval during which circulation is normalized, these patients have developed progressive respiratory distress (PRD), which has often led to death. The purpose of the following paper is to review the structural changes in the lungs in relation to the clinical course of the disease.

MATERIAL AND METHODS

The material has been derived from routine post mortem examinations at the Danderyds Sjukhus. The first case of PRD and advanced pulmonary lesions following shock was observed late in 1964. Since then the number of cases has increased to more than 30, of which 20 cases with adequate tissue material and clinical records are included in this study (Group I). All these cases required artificial ventilation with an Engstrom respirator and a high oxygen concentration. In addition, 12 cases in which death followed a period of mechanical artificial ventilation not associated with PRD have

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TABLE 1 *Cases of Progressive Respiratory Distress (Group 1) Clinical Data*

Case	Age & Sex	Basic Disease	Complications	Lowest Trc-count	Uraemia	Icterus
1	75 F	Haemorrhagic gastritis Gastrectomy	Postop haemorrhage, peritonitis pancreatitis	22 000	+	+
2	18 F	Meningococcaemia		6 000	+	+
3	41 F	Barbiturate intoxication	Pulm embolism	17 000	+	–
4	72 F	Bleeding ulcer Gastrectomy	Cardiac arrest Venous thromb	49 000	+	+
5	46 M	Multiple fractures Lung contusion	Haemothorax	80 000	+	–
6	34 F	Ileus Intestinal resection	Postop septicaemia.	30 000	+	–
7	58 F	Acute cholecystitis	Endocarditis Venous thromb	8 000	+	+
8	38 M	Ulcerative colitis Total Colectomy	Postop septicaemia. Venous thromb, pulm embolism	58 000	+	–
9	61 F	Gastric ulcer Gastrectomy	Postop peritonitis Venous thromb	140 000	–	–
10	21 F	Cholecystectomy	Stevens-Johnson syndrome	32 000	+	+
11	39 M	Strangulation ileus	Peritonitis septicaemia pulm embolism		–	–
12	41 F	Gastric ulcer Gastrectomy	Postop haemorrhage Possible primary renal disease	150 000	+	–
13	70 M	Acute pancreatitis	Peritonitis Venous thromb	86 000	+	+
14	22 M	Multiple fractures	Pneumothorax Septicaemia	33 000	–	+
15	42 F	Virus pneumonia?		74 000	+	–
16	78 M	Gastric ulcer Gastrectomy	Postop peritonitis	53 000	–	–
17	43 M	Acute pancreatitis	Peritonitis Venous thromb	117 000	+	–
18	20 F	Cirrhosis of the liver Spleno-renal anastomosis	Postop haemorrhage	31 000	–	+
19	78 M	Prostatectomy	Postop haemorrhage	16 000	+	+
20	29 M	Fracture of the femur	Fat embolism	166 000	–	–

TABLE 2 *Oxygen Treated Cases without Progressive Respiratory Distress (Group 2) Clinical Data*

Case	Age & sex	Basic Disease	Complications	Lowest Trc count	Uraemia	Icterus
21	37 F	Barbiturate intoxication		• •	+	–
22	63 M	Coronary disease	Pulm edema.	•	–	–
23	58 M	Hemorrhagic gastritis, Gastrectomy	Postop peritonitis porthal vein thrombosis	96 000	+	–
24	43 F	Traumatic rupture of the intestine	Duodenal fistula retroperitoneal abscess		–	–
25	75 F	Gastric cancer Gastrectomy	Mediastinitis Pulm embolism		–	–
26	44 M	Tetanus Curare administration	Cardiac arrest		–	–
27	71 F	Acute pancreatitis	Septicaemia		+	+
28	36 M	Acute pancreatitis	Peritonitis pneumonia pulm embolism		+	–
29	67 M	Acute pancreatitis	Peritonitis pneumonia	69 000	(+)	(+)
30	77 M	Acute appendicitis	Postop peritonitis aspiration, pneumonia.	18 000	+	–
31	50 M	Polyradiculitis with respiratory paralysis	Pneumonia septicaemia venous thromb	110 000	–	–
32	71 F	Coronary disease	Cardiac arrest pulm embolism		–	–

been analysed (Group 2). In 12 cases in Group 1 and 5 cases in Group 2 the lungs were inflated and fixed by infusing 5 % formalin into the bronchi at a pressure of about 50 cm. water. Whole lung paper mounted sections (Gough & Wentworth method) were produced in 8 and 2 of the cases, respectively. In 4 cases in Group 1, prior to fixing, a bronchography was made on one side and a pulmonary arteriography on the other, using a thick barium contrast medium. After the bronchography the lung was fixed by infusing formalin into the pulmonary artery. Sagittal slices 2–3 cm. thick were radiographed after fixation.

From the 17 cases in both groups where the lungs were fixed in the inflated position, 5–10 blocks of tissue representing all the lobes were taken for paraffin embedding and histological examination. In the other cases only 3–4 blocks of tissue from the lungs were available for examination. All the sections were stained with haematoxylin-eosin and Van Gieson's stain, a few also by the Ladewig method or with PAS. In 15 cases frozen sections were stained with Oil red O.

An approximate grading of the histological pulmonary changes was made as follows:

Hyaline membranes (HM): + = thin HMs in less than half of the histological sections; + + = thin HMs in more than half or thick HMs in less than half of the sections, or dissolving HMs; + + + = thick HMs in more than half of the sections.

Organization of HMs: + = infiltration of macrophages and inflammatory cells; + + = early to moderate proliferation of fibroblastic cells; + + + = HMs to a large extent dissolved and replaced by proliferating fibroblasts; + + + + = transformation to fibrous tissue.

Bronchopneumonia was graded from + to + + +, according to the distribution of inflammatory foci in the sections. Interstitial oedema and increased cellularity: + = moderate, + + = pronounced. The presence of microthrombi and fat emboli was not graded.

RESULTS

1. Clinical Background

The 20 patients in Group 1 were treated for various serious diseases and were transferred to the intensive care unit because of acute complications leading to circulatory failure and shock. 9 patients had postoperative complications such as bleeding, sepsis or peritonitis, 3 had trauma with fractures, 2 had acute pancreatitis, 2 had acute infections and the remaining 4 cases involved intoxication, haemetemesis, acute cholecystitis and Stevens-Johnson syndrome, respectively. Most of the traumatic cases are transferred to the Dept. of Forensic Medicine for post mortems which is the reason why so few are included in this study. Besides circulatory failure all the patients likewise showed signs of respiratory insufficiency which required a tracheotomy and artificial ventilation, initially with little supplementary oxygen. As a rule the circulation could be restored by means of modern shock treatment, but after a few days the patients developed PRD which, combined with other complications such as anuria, jaundice and sepsis, resulted in death. In cases 9, 15 and 20 the development of PRD preceded the administration of mechanical artificial ventilation. During the progressive phase the oxygen concentration, the ventilatory volume per minute and the inspiratory pressure had to be raised step by step, often to maximum values, in order to maintain the arterial oxygen tension at an acceptable level. Thrombocytopenia was a common and significant finding here. In 19 cases the thrombocyte counts were known and 14 of these presented less than 80 000 thrombocytes (Table 1).

Parallel with the development of PRD the chest films revealed widespread coarse flecks and blurred shadows which later merged into massive consolidation. Before this the chest films were normal or showed only basal atelectasis, except in the cases of pulmonary contusion, pneumothorax and sepsis where these changes were visible. In Case 15 the patient developed non-characteristic respiratory symptoms and a roentgen picture seemed to indicate virus pneumonia. After a period of improvement both clinically and roentgenologically, PRD developed, the clinical and roentgenological pictures being the same as that in the other cases.

In addition these patients were given complex treatment including heparin, sedation, transfusion of blood or plasma, intravenous infusion of fluids and electrolytes, steroids, antibiotics, and in several instances peritoneal dialysis. The scheme of treatment of these patients at the Danderyds Sjukhus has been presented in another paper, (17).

TABLE 4 *Oxygen Treated Cases without Progressive Respiratory Distress (Group 2) Clinico pathological correlation*

Case	Artificial ventilation			Lung pathology					
	Duration of days	Max oxygen conc %	Max resp vol L/min	Hyaline mem branes	Organ ization of HM	*Broncho-pneum.*	Inderst oedema & inflam	Micro-thrombi	Fat emboli
21	$2^1/_2$	50	10	−	−	+	−	+	
22	$1^1/_2$	65	11	+ +	−	−	+	−	
23	$1^1/_2$	?	?	+	−	−	−	+	−
24	17	80	18	+	−	+ +	+	−	
25	5	80	12	+	−	−	−	+	
26	6	25	16	+	−	+ +	+	+	
27	9	65	14	+ +	−	+	+	+	
28	29	50	20	+ +	−	+ + +	+ +	−	
29	7	80	25	+ +	−	+ + +	+ +	+	+
30	11	90	22	+ + +	−	+ + +	+ +	+	−
31	34	80	20	+	+	+ +	+	+	−
32	9	50	?	+ +	+	−	+	−	

the different parts of one and the same lung *The earliest lesions seemed to be alveolar and interstitial oedema and thickening of the alve olar walls due to capillary congestion diapede* sis of erythrocytes and increased cellularity (Fig 3) Alveolar haemorrhage *was also* usu al In all cases but one HMs of varying thick ness and extent were present In several cases in group 2 the HMs were sparse and thin and probably *an early phase* of develop ment while widespread *thick HMs were seen* in most cases in Group 1 *The HMs* were located mainly in the respiratory bronchioles and alveolar ducts a fact which was *clearly* vi ble in lungs fixed in the inflated position These membranes *appeared* first on the tips of the alveolar walls along *the ducts* (Fig 2), but gradually they merged and outlined *the* alveolar ducts appearing to seal *off* the al veolar sacs The HMs could be as much as 100 microns thick In the areas with HMs hypertrophy proliferation and desquamation of the alveolar lining *cells and* an accumula tion of macrophages in the al*veolar* sacs and along the membranes was also *discernible* (Fig 3 In many cases macrophages infil trated the membranes sometimes they formed giant cells and seemed to initiate the resorp tion of the hyaline material

In cases where the patients survived *for* longer periods, a proliferation of fibroblastic cells within the HMs was observed *while at* the same time the hyaline material was gradu ally resorbed (Fig 4) These fibroblastic cells evidently derived from the macrophages Pro-liferating fibroblasts with remnants of hyaline material around the lumina of the respiratory bronchioles and alveolar ducts were a very characteristic lesion which is probably specific to these cases (Fig 5) In a few cases colla gen formation was observed (*Fig 6*) and in *case 18* many of the respiratory bronchioles and alveolar ducts were filled with newly formed cellular fibrous tissue (*Fig 7*)

In most of the cases inflammatory lesions mainly broncho pneumonia, but also inter stitial inflammation were observed In some cases organization of an alveolar exudate oc curred parallel with the organization of the HMs Pronounced interstitial oedema with dilatation of the lymphatics and fibroblastic proliferation in the interlobular septa and around the large vessels and bronchi was also a characteristic component of the histological picture

Microscopic thrombi in the small muscular pulmonary arteries and arterioli were present in 16 of the cases *in* Group 1 and in 8 of the

TABLE 3 *Cases of Progressive Respiratory Distress (Group 1) Clinico pathological correlation*

Case	Artificial ventilation				Lung pathology					
	Duration of, days	Survival with PRD days	Max oxygen conc %	Max resp vol L/min	Hyaline mem branes	Organ-ization of HM	Broncho pneum	Inderst oedema & inflam	Micro thrombi	Fat emboli
1	5	4	70	16	+ +	–	–	–	+	
2	12	2	90	22	+ + +	–	+ + +	+	–	
3	8	4	100	20	+ + +	–	+ +	+	+	
4	9	3	60	18	+ + +	–	+ +	+ +	+	+
5	8	5	80	19	+ + +	+	+	+ +	+	–
6	6	5	90	26	+ + +	+	+ +	+ +	+	
7	16	4	90	20	+ +	+	+ +	+ +	+	+
8	13	6	100	30	+ + +	+	+ +	+ +	+	
9	3	3	100	30	+ +	+	+	+	+	–
10	13	5	90	20	+ + +	+	+ +	–	–	–
11	$9^1/_2$	$6^1/_2$	90	22	+ +	+ +	+ +	–	+	+
12	9	6	80	20	+ + +	+ +	+	+	–	
13	11	8	60	18	+ +	+ +	+	+	+	+
14	11	6	80	25	+ +	+ + +	+ +	+	+	
15	9	9	85	23	+ + +	+ + +	+	+	+	+
16	13	9	80	26	+ +	+ + +	+ +	+ +	+	–
17	21	14	100	30	+ +	+ + + +	+ +	+ +	+	–
18	44	40/8	90	23	+ + +	+ + + +	+ + +	+ +	–	
19	14	$10^1/_2$	80	27	+ +	+ + + +	–	+	+	
20	11	11	90	24	+ + +	+ + + +	+ +	+	+	+

The 12 cases in Group 2 were also treated in the intensive care unit and required mechanical ventilation for 1–34 days In these cases there was a fairly stationary respiratory function without any final development of PRD In 8 cases shock treatment was administered as in Group 1, i e 3 on account of post-operative complications, 2 on account of acute pancreatitis and the remaining 3 on account of intoxication, heart failure with irreversible pulmonary oedema, and cardiac arrest, respectively Furthermore 2 patients required artificial ventilation because of respiratory paralysis, one a case of tetanus treated with curare, the other a case of polyradiculitis The 2 other patients in this group required artificial ventilation for massive pneumonia (Table 2) The oxygen concentration and the ventilatory volume varied greatly within this group (Table 4)

In Group 2 there were only sporadic pulmonary x-ray examinations These showed atelectasis, pneumonia or normal conditions, but none of the characteristic lesions which were observed in Group 1 Uraemia occurred in 5 cases, jaundice in 2 cases Thrombocyte counts were made only in 4 cases, 2 of which presented values below 80 000

2 *Pathology*

The pathological-anatomical pulmonary changes were of the same nature in all the patients, though of varying degree In group 1 the changes were without exception advanced, while in Group 2 they varied more The lungs were heavier than normal, of a firmer consistency and had a reduced air content The cut surface was dark red to pale greyish and often showed focal haemorrhage and bronchopneumonia, particularly in the dorsal parts The dark red areas were oedematous and congested while the greyish areas were drier, solid and brittle The extent of the changes could be studied well in macrosections and even better in bronchograms As a rule the central, dorsal and basal parts of the lungs were most consolidated (Fig 1)

The histological picture varied likewise in


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Fig 1 a/ Macrosect on b/ bronch ogram of left lu g of case 17 sl o v g mass ve cer tral co sol dat on

cases in Group 2 (Fig 9) It cannot be determined whether this was the result of local intravascular coagulation or embolization from thrombi in the systemic veins Macroscopical pulmonary emboli and/or venous thrombi occurred in 8 cases in Group 1 and in 5 cases in Group 2 The pulmonary arteriography was normal in 2 cases unsatisfactory in 1 case and in one case it showed scattered emboli Fat emboli in the alveolar or renal capillaries were found in 6 out of 11 cases examined in Group 1 and in 1 out of 4 cases examined in Group 2 Fat in the alveolar macrophages was a common occurrence As regards the other organs degeneration and minor necrosis of the adrenal cortex was observed in many cases also osmotic nephrosis or tubular necrosis of the kidneys and parenchymatous degeneration of the liver were noticed Myocardial necros s was observed in 2 cases and in 8 cases there was endocarditis or other septic lesions

3 *Clinico pathological Correlations*

Tables 3 and 4 show that patients who required large ventilatory vol imes and l gl oxygen concentrations generally revealed severe lung lesions at the post mortem th s was particularly true of the HMs B it exten sive bronchopneumonia also increased the oxygen demands as in cases 29 and 30 which

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Fig 2 Early l yal ne n eml ranes in alveolar d ct form ng on tij s of al eolar sej ta Case 26 Haematoxyl n eos n 135 ×

Fig 3 Hyal e membra es in al eolar ducts partly f ll ng adjacent al eolar sacs Acc n lat on of n acrophages Al eolar septa tl ckened d e to oe lema and increase l cellularity Case 22 van Gieson 335 ×

Fig 4 Transversely c t al eolar d ct l ne l l y d solv ng membrane i filtrated by i flai matory an l f brol last c cells Case 11 l ade g 150 ×

Fig 5 Al colar duct l ne l l y prol ferat ng f l roblast c cells an l remnants of l yal ne material partly f ll ng adjacent alveolar sacs Case 15 Haematoxyl n eo n 150 ×



required ventilatory volumes of more than 20 litres/min and an oxygen concentration of about 80 per cent On the other hand a high oxygen concentration was not always followed by the development of extensive HMs Patient no 24 had breathed 70 80 per cent oxygen for 3 days patient no 25 breathed 80 per cent oxygen for 20 hours and patient no 31 had 80 per cent oxygen for 4 days In these 3 cases there were only occasional thin HMs in the lungs Neither was the development of HMs definitely related to the duration of mechanical artificial ventilation In cases 24 and 31 there were only sparse HMs after 17 and 34 days artificial ventilation respectively, while in case 9 the HMs were relatively extensive after ventilation for 3 days

In Group 1 there was a certain relation between the period of survival after onset of PRD and the stage of organization of the HMs There was no organization at all in cases 1–4 in which PRD developed during the 2–4 final days In patients nos 5–10 who survived for 3–6 days after the onset of PRD the membranes were infiltrated by macrophages in patients nos 11–16 who survived for 6–9 days there was proliferation of fibroblastic cells and in patients nos 17–20 who survived for 10–40 the HMs were partly replaced by fibrous organization tissue Forty days before the death of patient no 18 severe respiratory distress developed which however responded to therapy and improved temporarily Eight days before death the respiratory difficulty recurred and the condition deteriorated rapidly There were likewise two stages of histological lesions some of the alveolar ducts and respiratory bronchioles were obliterated by newly formed fibrous tissue while others were lined by HMs showing early organization As regards the cases in Group 2 there was practically no organization of the HMs at all

According to expectation extensive bronchopneumonia was more usual the longer the period of treatment Interstitial inflammation seemed not to be related to sepsis

Thrombocytopenia occurred in 14 cases in Group 1 and in 2 cases in Group 2 Thrombocytopenia in connection with shock is considered to be secondary to intravascular coagulation Microthrombi the pathogenesis of which has not been proven were encountered in 75 per cent of the cases in both Group 1 and Group 2 The frequency of venous thrombi and macroscopic pulmonary emboli (about 40 per cent) however, was not above that to be expected in seriously ill patients Microscopic fat emboli were encountered in 6 out of 11 examined cases in Group 1, only 1 of these patients had fractures In Group 2 staining for fat was made only in some cases

DISCUSSION

It is only in the last 10 years that attention has been drawn to the development of pulmonary lesions with HMs in adult critically ill patients (7, 12 19) The macroscopic picture is characteristic The lungs are heavy and more or less consolidated with focal haemorrhage and bronchopneumonia but without atelectasis Histologically these lesions are characterized by capillary congestion interstitial and intra alveolar oedema focal haemorrhage hyperplasia and hypertrophy of the alveolar lining cells accumulation of alveolar macrophages and the formation of HMs By increasing the alveolar capillary barrier against gas diffusion these lesions constitute the morphological basis for respiratory distress The HMs are located mainly in the alveolar ducts and respiratory bronchioles and less often in the alveolar sacs This can be difficult to see in routine sections but it emerges clearly in lungs fixed in the inflated position or in sections stained for elastin (2 18)

Fig 6 Alveolar duct lined by fibroblastic cells with early collagen formation sealing off surrounding alveolar sacs Case 19 Haematoxylin eosin 127 ×

Fig 7 Respiratory bronchiole and alveolar ducts filled by young cellular fibrous tissue Case 18 van Gieson 135 ×

Fig 8 Alveolar duct completely obliterated by fibrous tissue Thickening of surrounding alveolar walls van Gieson 132 ×

Fig 9 Microthrombi in small pulmonary arteries Case 15 Ladewig 370 ×


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that concentrated oxygen is the direct cause of pulmonary lesions in man is the fact that a higher concentration of oxygen is not administered until diminishing arterial oxygen tension demands this in other words respiratory insufficiency is established before the oxygen toxicity enters into the picture (1) In some instances the characteristic shadows on the chest films have been observed before the administration of mechanical ventilation with supplementary oxygen (case 9) Neither does a high concentration of oxygen always result in pronounced pulmonary lesions (cases 24 25 31)

Another factor common to these patients is initial circulatory failure with diminished tissue perfusion As a rule modern intensive care restores the circulation but after a certain period PRD develops and increasing infiltrates are visualized on the chest films The pathophysiology of the pulmonary circulation after treatment for shock has been studied on experimental shock lung models If hypovolaemic shock is induced through bleeding and after a few *hours the circulation* is restored by means of reinfusion pulmonary vascular resistance increases during the following 3 days and the alveolar perfusion decreases (14) This in its turn is followed by massive atelectasis and transudation However total ischaemia throughout a corresponding period of time does not cause damage to the lung which seems to indicate that the injury is caused by high functional demands during a low perfusion state (23) In shock models of this type damage to the lungs is more frequent than to any other organ (24) Following experimentally induced sepsis the same vasomotorial effects and structural lesions have been observed in the lungs of animals (11)

Both in animal experiments and in patients with respiratory distress a reduction of alveolar surfactant has been observed This reduction is probably caused both by impaired phospholipid synthesis on account of diminished perfusion and by the inactivating effect of alveolar transudate (13 15) Physiological examinations of newborn children with respiratory distress also indicate that this condition is connected with vasoconstriction and alveolar hypoperfusion, (10) At Danderyds Sjukhus the entire complex of organ complications following shock is called the hypoperfusion syndrome , which term apart from pulmonary lesions also embraces symptoms of renal hepatic adrenal and cerebral injuries and disturbance of the coagulation fibrinolysis system with thrombocytopenia Besides vasoconstriction and diminished perfusion it is probable that intravascular coagulation is also an important factor (17) Impaired fibrinolytic activity has also been discussed (7)

At present the most likely view on the pathogenesis of progressive respiratory distress as a complication of shock is that the process is caused by pulmonary vasoconstriction and diminished alveolar perfusion intravascular coagulation and a reduction of alveolar surfactant to which may be added factors such as the toxic effects of oxygen the mechanical effects of the respirator infection of the respiratory tracts via the tracheostoma general infection over administration of fluid cardiac insufficiency etc (1 12 19)

Pulmonary lesions with HMs occur in various diseases such as radiation pneumonitis uraemia severe pulmonary oedema viral and bacterial pneumonia the inhalation of certain gases and hyaline membrane disease in the newborn In these conditions the gas diffusion in the lungs is impaired by interstitial oedema and alveolar exudation with the formation of membranes This leads to increasing respiratory insufficiency which in its turn calls for more oxygen When the patient dies after a period of PRD the lung changes can scarsely be distinguished from the pulmonary complications following shock The respiratory distress lung is thus a more suitable term than the often used "respirator lung which in fact relates only to one of several factors in a limited group of patients.

Supported by a grant from *Vitrum Ltd* Stockholm Sweden

If the patients with PRD survive for more than 5–6 days a very characteristic picture develops in that the HMs are gradually re sorbed and replaced by proliferating fibro blastic cells which *probably derive from al* veolar macrophages This organization tissue seems to seal off the alveoli from the alveolar ducts After about 14 *days* collagen appears and cellularity diminishes In cases where the patients survive for a long time, newly formed fibrous tissue can fill entirely many of the alveolar ducts a picture which is probably specific for this condition

Following a period of artificial ventilation HMs may be found in the lungs even if PRD has not occurred as in Group 2 of this study Half of these patients had only sparse thin HMs probably in an early phase of develop ment while abundant HMs were found mainly in cases where the clinical and the pathological pictures were obscured by mas sive pneumonia Organization of the HMs was practically absent in all patients in this group despite often extensive periods of treat ment This may be because the HMs in these cases did not form until shortly before death possibly as a result of terminal cardiac insuf ficiency It is also possible that the abnormal conditions in the respiratory tracts of patients in Group 1 *i e* high inspiratory pressure with positive intrathoracic pressure and high oxy gen concentration stimulated fibroblastic pro liferation *Regele* (23) points out that the positive intrathoracic pressure during me chanical ventilation impairs the lymph drain age from the lungs and leads to chronic lym phoedema with proliferation of mesenchymal cells and delayed resorption of exudate *Northway et al (21)* who have studied the later stages of hyaline membrane disease in the newborn calling it bronchopulmonary dysplasia consider that the lesions can be reversible even if the chest films show diffuse changes and the respiratory symptoms may have been present for a long time On the other hand *Regele* (23) describes an adult pa tient in whom diffuse interstitial fibrosis much reminding of the Hamman Rich lung deve loped after 10 weeks of mechanical ventila tion At Danderyd Sjukhus we have seen only one case which at the post mortem was inter preted as lung fibrosis after recovery from respiratory distress (this case is not included in the two patient groups)

The patient in question was a 4 year old boy who was *admitted to the intensive care unit for* virus pneumonia involving PRD After 14 days in a respirator with 70 per cent oxygen and an in spiratory pressure of more than 50 cm water there was an improvement the roentgen picture celared weaning was attempted and after another two weeks the patient was off mechanical ventilation He died 18 days later from acute pulmonary haemorrhage Histological examination of the lungs showed fibrous thickening of the interlobular septa and also focally of the alveolar walls which was reminiscent of the Hamman Rich lung Moreover several respiratory bronchioles and alveolar ducts were totally obliterated by cellular fibrous tissue while the surrounding alveoli were partly collapsed (Fig 8)

A factor common to critically ill patients who develop PRD is that they require the administration of supplementary oxygen if they are to survive usually via a respirator and in high concentration In post mortem in vestigations a relationship has been established between the severity of histological pulmonary lesions and the prolonged use of artificial ventilation with a high concentration of oxy gen It has been claimed that this indicates a causal connection (2 8 20 23) Animal ex periments have shown *clearly that exposure to* a high concentration of oxygen for a number of days can cause damage to healthy lung tissue also that susceptibility to oxygen varies among different species (4 5 9) The effect of the oxygen can be raised by substances known to influence vasomotorial functions (6) The experimentally induced lesions in animals are in many respects similar to those found in human subjects who have received oxygen therapy (22) Electron microscopic studies have shown that the initial lesion con sists of interstitial oedema in the alveolar walls and this is *followed by visible structural lesions* in the capillary endothelial cells The alveolar lining cells and macrophages are not affected (3 16) An argument against the hypothesis

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ACTINOMYCES IN TONSILLAR TISSUE

A Histological Study of an Autopsy Material

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At autopsy *Actinomyces* colonies were found in the tonsils in 9 out of 22 bodies under the age of 35 years This seems to indicate that tonsillar crypts is a normal habitat of such colonies

In a tonsillectomy material, *Actinomyces* colonies have previously been found in 17 out of 103 cases (*Gruner* 1969) The significance of these colonies was uncertain, however and their prevalence in normal tonsils remained unknown Therefore an autopsy material has been surveyed

MATERIAL AND METHODS

Tonsils from 22 bodies have been examined Table 1 lists the underlying causes of death Because primary malignant disease of the lymphatic system may affect the tonsils such cases were excluded Table 2 gives the age and sex distribution Cases above 37 years of age were excluded, this being the maximum age of patients in the previous tonsillectomy material (*Gruner* 1969)

The organs were taken out en bloc by mortuary technicians who also removed the tonsils These were fixed in 4 per cent formaldehyde solution for at least 24 hours and afterwards a central slice was taken from each tonsil and embedded in paraffin Two sections were studied one stained with haematoxylin and eosin the other with periodic acid *Schiff* (PAS) When colonies were found, still another section was stained with *Gram*

Mainly due to *rigor mortis* the removal of tonsils failed at about every third attempt, and in two cases only one tonsil was taken out Previous unrecorded tonsillectomy may have been responsible for this failure The present material is therefore not the total number of cases eligible, but may be considered unbiased

Identification was made according to the same criteria as previously used (*Grüner* 1969) The typical picture of the PAS positive and *Gram* positive 'ray fungus' whose *hyphae* have club-like ends discernible in good sections with no shrinkage

Received 20 vii 70

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RESULTS

In all cases, bacterial colonies and cellular debris were found in the crypts *Actinomyces* colonies were found in the tonsils of 9 among the total of 22 bodies No significant correlation between the cause of death and *Actinomyces* colonies was found (Table 1) The great proportion of colonies in cases of sudden death should be noticed as should also the lack of colonies when malignant tumour

TABLE 1 Actinomyces *Colonies According to Underlying Causes of Death*

	Number of cases	Colonies
Accidents	8	4
Recent coronary thrombosis	2	2
Malignant tumour	6	0
Infection	1	1
Mental disease or disease of the nervous system	5	2
Total	22	9

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BRIEF REPORT

SKIN INVOLVEMENT IN SECONDARY AMYLOIDOSIS

Per Westermark

Secondary amyloidosis is a not unusual complication of rheumatoid arthritis (RA) Amyloidosis of this form is a serious condition, especially because of renal involvement In autopsy series of RA the frequency of amyloidosis has been found to be up to 60 per cent (7), while that in randomly selected living RA patients is much lower (1)

In earlier years, the diagnosis of generalized amyloidosis was made mainly with the aid of the Congo red test and later by biopsies from different organs mainly the gingiva, liver and kidney (4) Since the 1960s biopsy of the rectal mucosa has been the predominant diagnostic method (1, 3) The aim of the present study was to determine the occurrence of amyloid in clinically normal skin in cases of secondary, generalized amyloidosis

Material and Methods

All autopsy cases with a clinical diagnosis of RA were studied during several different periods Specimens from different organs, including the rectum and abdominal skin, were taken for examination These were fixed in 10 per cent formalin and 10 per cent buffered neutral formalin, embedded in paraffin and sectioned The sections were then stained with alkaline Congo red (5) and examined in a polarization microscope



Fig 1 Sweat glands surrounded by rings of amyloid Alkaline Congo red Polarization microscope Approx × 600

Results

During a total period of 7½ months, 625 adult patients were autopsied and among these 22 had a clinical diagnosis of RA (3 5 per cent) In 5 (23 ne [illegible]
arth [illegible]
type [illegible]
amy [illegible] been diagnosed during life Accumulations of amyloid especially in blood vessels, were observed in all internal organs examined including blood vessels in the rectal mucosa, in these 5 patients

In 4 of the amyloidosis patients, but not in the 5th, amyloid deposits were found in the skin The skin lesions followed a characteristic pattern The upper parts of the corium were normal Around several sweat glands and occasional sebaceous glands and hair follicles, at the borderline between the corium and the subcutaneous tissue, thin rings of amyloid were found between the basal membrane of the adnexa and the connective tissue Here and there very thin amyloid rings were found also around fat cells These changes were not general and a number of areas were quite normal

Discussion

The presence of amyloid in the skin of patients with secondary amyloidosis was observed at an early date (6) The method of examination was

Received 20 xi 70 *from Department of Pathology University of Uppsala, Uppsala, Sweden*

TABLE 2 Actinomyces *Colonies According to Age and Sex*

Age in years	Males Cases	Males Colonies	Females Cases	Females Colonies	Total Cases	Total Colonies
Under 10	1	1	2	0	3	1
10–14	1	1	0	0	1	1
15–19	5	2	2	1	7	3
20–24	5	1	1	1	6	2
25 29	1	1	2	1	3	2
30–34	2	0	0	0	2	0
Total	15	6	7	3	22	9

was the cause of death The age and sex distribution does not show any definite trend (Table 2) Colonies were found in 7 out of 18 bodies above the age of 15 years

COMMENT

In the present autopsy material the proportion of *Actinomyces* colonies was apparently greater than that in the previously reported tonsillectomy material (*Gruner* 1969) The age distribution in this tonsillectomy material was different in that the number of children was greater Nevertheless, after the age of 15, the present proportion 7/18 (38 9 per cent) may possibly be compared with a proportion of 13/60 (21 7 per cent) in the tonsillectomy material By the *Fisher Irwin* test (one tailed) this difference is significant (P = 0 04)

Actinomyces is considered an "opportunistic' microbe (*Peabody* & *Scabury* 1960 *MacKinnon* 1962, *Utz* 1962) Therefore it was surprising that no colonies were found when malignant tumour was the cause of death

Wilkinson (1929) found colonies in only 1 8 per cent of tonsils surgically removed while the prevalence was 16 5 per cent in the tonsillectomy material (*Gruner* 1969) The reason for this difference was not clear, but it was suggested that this greater proportion might be due to the fact that criteria for tonsillectomy in recent years are stricter than they were in the Late Twenties If *Actinomyces colonies* were a sign of tonsillar disease then a greater proportion would be expected when fewer normal tonsils were removed

The present great proportion in cases of sudden death seems to invalidate these speculations and another explanation must be *sought* The method of removal was probably different in the materials of *Wilkinson* (1929) and *Gruner* (1969) and these materials again differend from the present Previously, tonsillectomy was performed by a tonsillotome by now, dissection is the current method *In the present material the tonsils* were hardly subjected to squeezing at all This may account for an even greater proportion than in the tonsillectomy material (*Gruner* 1969) as some pressure on the tonsillar tissue during tonsillectomy is unavoidable The frequent finding of *Actinomyces* colonies in the orifices of crypts may support this hypothesis Anyhow, the present material seems to indicate that tonsillar crypts is a normal habitat of *Actinomyces* colonies

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Acta path microbiol scand Section A 79 81-90 1971

INHIBITORY EFFECT OF DEAE-DEXTRAN ON TUMOUR GROWTH

3 *Effect of Charge Density and Molecular Size*

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Head J Bichel, M D

The effect of different DEAE-dextran derivatives on the surface charge of tumour cells has been investigated The DEAE-dextran varied in molecular weight and charge density (degree of substitution with diethyl amino-ethyl groups) It was shown that the binding to the tumour cell surface and the ability to neutralize the negative charge of the cell surface was dependent upon the molecular weight and the charge density Higher molecular weight and higher degree of substitution gave a stronger binding The toxicity of the various compounds in mice and amoebae was correlated to the cell surface affinity The biological effect of the different drugs was investigated in the C3H mice using the transplantable ascites tumour JBI which is a plasmocytoma The DEAE dextran has an inhibitory effect on the growth of transplanted tumours This effect was greatest for the compound with the highest molecular weight (2 mill) and the highest degree of substitution (50 per cent), and was negligible for a compound with molecular weight of 64 000 and 13 per cent substitution

Basic polymers are strongly bound by cell surfaces This property of basic polymers may be essential in several of their biological effects on cells Synthetic linear polymers of lysine and ornithine have been shown to have a strong inhibitory effect on bacterial growth (*Katchalski et al* 1952) and on coliphages T2 and T4 (*Shalitin et al* 1962) A similar growth inhibiting effect has been demonstrated on tumour cells Incubation „in vitro" of tumour cells with polylysine or with lysine rich histones decreases the growth rate of mammary tumours in mice (*Shah & Reilly* 1967) Complete inactivation of the transplantibility of a mouse leukaemia NJA in C3H/A mice is obtained after incubation „in vitro" of the tumour cells with the soluble basic ion exchanger diethylaminoethyl dextran, (DEAE-D) molecular weight 2 000,000 and containing one positive charge per two glucose units The inhibitory effect of DEAE-d is reversed with heparin (*Larsen & Olsen* 1968) and dextran sulphate (*Larsen & Thorling 1969, Thorling & Larsen* 1969) This principle is outlined in Fig 1 Presumably the inhibitory effect of DEAE-d is dependent upon the ability of polycations to interact with the tumour cell *Ryser* (1967) has suggested that the biological effect of basic polymers is associated with their molecular structure

The purpose of the present study has been to investigate the influence of structural factors of DEAE dextran on the binding to

Received 6 vii 70

not very specific, however, and these results have therefore been doubted In a recently published study involvement of the skin in secondary amyloidosis is considered to be rare (2), an opinion which would seem to be contradicted by the present findings No clinically observed skin changes which might be attributable to the amyloidosis were noted, however

In order to establish the diagnosis in generalized amyloidosis, rectal biopsy, which is a reliable method, is mainly employed nowadays Some cases arise, however, in which rectal biopsy is difficult for one reason or another It is possible that abdominal skin biopsy, which is harmless to the patient and easy to performe, could be used as an alternative diagnostic method in such cases

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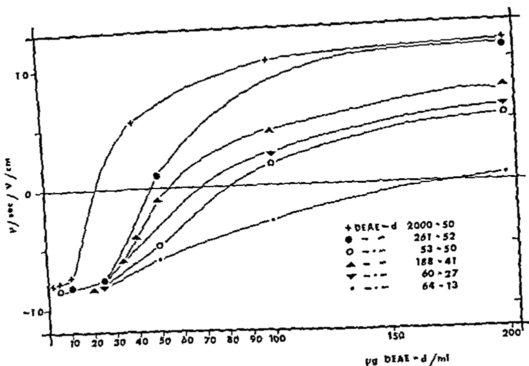


Fig 2 De ... ncentrations of the different DEAE dextran derivati ... and then degree of substitution in per cent e g 200 ... with di-ethyl amino-ethyl groups

4 2 *Results of Cell Electrophoresis*

Binding of DEAE d to Tumour Cells

The influence of DEAE-d on the electrophoretic mobility of JBI tumour cells was studied in phosphate buffered saline pH 7 4 added to 5 per cent sorbitol in a ratio of one volume PBS to four volumes of 5 per cent sorbitol As seen in Fig 2 the initial negative mobility of the JBI tumour cells of -0.8 ± 0.1) µm/sec volt/cm is reduced upon addition of DEAE-d As the cellular mobility approaches zero a high degree of cell aggregation is noted, but a higher DEAE-dextran concentration at which a positive cellular mobility is observed, the degree of aggregation is reduced It is seen that high molecular weight DEAE-d 2000/50 has the highest affinity for JBI tumour cell surfaces and low molecular weight DEAE-d the lowest affinity of the substances tested A relative expression of the affinity of the various DEAE dextrans for the JBI tumour cell surface may be obtained by comparing the amount of polycations necessary to furnish

... qua ... obility, the number of positive groups adsorbed per cell must be identical in order to obtain a given mobility If zero mobility is chosen, the relative affinities of the DEAE dextrans may be expressed as shown in Table 1

B 1 Autoradiography

Tritiated DEAE-d 2000/50 was prepared by Amersham England by a catalytic exchange procedure and purified by collecting the fraction with the same void volume as Blue Dextran 2000 from a Sephadex G 200 column The product was then treated with a small quantity of charcoal to remove traces of discolouration The freeze-dryed product had a specific activity of 0 029 mCi/mg

JBI tumour cells were washed twice in PBS buffer and incubated in buffer containing 1–5 mg/

6*

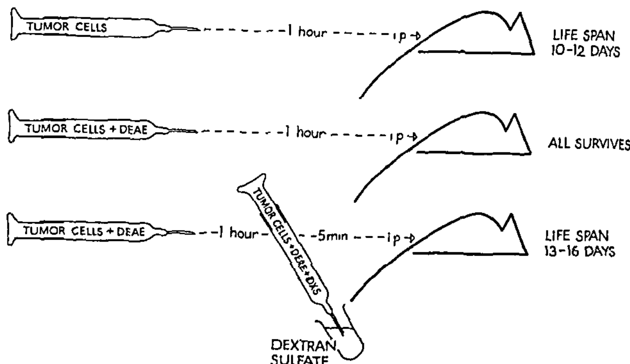


Fig 1 Basic principle for „in vitro" reversibility of the DEAE inhibition of tumour cell growths

tumour cell surfaces and compare the binding affinity to biological activity of the polymers

MATERIAL AND METHODS

Tumour Cells and Mice

The JBI tumour grown as an ascitic tumour in C3H/A mice was used Originally this tumour arose spontaneously in AK/A mice (Bichel 1951) It has been maintained as ascitic tumour in the C3H/A mice since 1955 The C3H/A mice being C3H mice maintained since 1950 by sister brother inbreeding in this laboratory The Lissamine dye exclusion test was used to check the viability of the tumour cells prior to inoculation

MATERIALS

A series of DEAE dextrans of different molecular weights and charges was kindley supplied by Dr Wolfgang Richter Pharmacia, Uppsala The following DEAE dextrans were used in the study DEAE d, 2000/50 *i e* DEAE-dextran Mw 2 000 000 50 per cent Diethylaminoethyl substitution per glucose unit, DEAE d 261/52 DEAE d 53 5/50, DEAE d, 188/41, DEAE d 60/27, and DEAE d, 64/13, and Acetyl amino ethyl dextran Mw 96 000, 16 per cent substitution Dextran sulphate sodium salt prepared from dextran with Mw 2 000 000 and 17 ± 0 5 per cent sulphate The Neuraminidase used was prepared by Behringwerke from Vibrio cholerae

A 1 Cell Electrophoresis

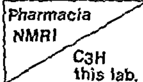
Cellular electrophoretic mobilities were measured at 25° C ± 0 1° C in a rectangular measuring cuvette (*Fuhrmann & Ruhenstroth Bauer* 1965) Zytopherometer Carl Zeiss Contamination with heavy metal ions from the copper copper sulphate electrodes was abolished by the use of a bridge of fluorated gelatin (Schwartz Bioproduction) A small amount of potassium ferricyanid was incorporated into the gel in order to be able to trace contamination with copper

Tumour cells and normal human erythrocytes were prepared for electrophoresis by washing three times in phosphate buffered saline (PBS) pH 7 4 and once in PBS sorbitol (1 4) (1 vol PBS 4 vol sorbitol 5 per cent) buffers were prepared from glass destilled water) After resuspension in PBS sorbitol the cells are kept by 0 2° C until use The times for cells to traverse 31 or 124 µm in both directions at a current of 3 0 m amp were recorded In the standard procedure 20 cells were measured in both directions by each concentration of polyelectrolytes Tumour cells were taken from donor mice inoculated 6 10 days earlier and human erythrocytes were used as a reference to check the cell electrophoresis apparatus

TABLE 2 *Acute Toxicity of Various DEAE Dextrans*

Mw x 10³ / %Substitution	100 mg/kg	200 mg/kg	400 mg/kg	800 mg/kg	1600 mg/kg	3200 mg/kg	6400 mg/kg	12.800 mg/kg	LD50 mg/kg
64/13	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0	0 / -	1 / -	12.800
60/27	0 / 0	0 / 0	0 / 0	0 / 0	2 / 0	3 / 2	3 / 3	3 / -	2000
188/41	0 / 0	0 / 0	0 / 0	0 / 2	3 / 1	3 / 3	3 / -	3 / -	1000
535/50	0 / 0	0 / 3	0 / 1	3 / 3	3 / 3	3 / -	3 / -	3 / -	550
261/52	0 / 1	0 / 2	3 / 3	3 / 3	3 / -	3 / -	3 / -	3 / -	200
2000/50	1 / 1	3 / 3	3 / 3	3 / -	3 / -	3 / -	3 / -	3 / -	110

0 *no deaths*
1 *one dead out of 3 mice*
2 *two dead out of 3 mice*
3 *all dead*



the label could be removed by neuraminidase and partly by dextran sulphate Cells previously treated with neuraminidase and subsequently incubated with labelled DEAE dextran would also b*ind the DEAE dextran* In these instances it is supposed that the DEAE dextran is bound to other acid groups on the cell surface which have been exposed after removal of the sialic acid component of the mucoproteinlayer (*Larsen* & *Thorling* 1970)

C I *Toxicity Determinations in Mice*

The acute toxicity was determined as the LD 50 (*dead within 24 hrs*) by *A/B Pharmacia* in NMRI mice and repeated in the C3H *strain of mice in our lab. The appropriate* amount of the various preparations of DEAE dextrans were given i p in phosphate buffered saline pH 7 4 As shown in *Table 2 there is fairly good agreement* between the two sets of determinations The LD 50 used here is based on the combined data.

In Fig 4 the correlation between *the toxicity* of the various compounds and their ability to change the surface charge of erythrocytes and JBI ascites tumour cells is *shown It appears that for compounds* with near the same degree of DEAE *substitution*, the toxicity is linearly correlated *to the cell* surface affinity in a double log system It *is seen that the greater the* ability is to change the negative surface charge of *the cells*, the greater the toxicity It also appears that the *molecular* weight of the derivative is decisive for the effect the higher the *molecular* weight the greater the surface effect and toxicity The two compounds (60/27 and 64/13) *with about* the same molecular weight

TABLE 1 *Concentration of DEAE dextrans at Which Zero Mobility for JBI Tumour Cells Is Obtained §*

DEAE dextran Mw × 10³	% substitution	µg/ml added of zero mobility*
2000	50	22
261	52	45
188	41	55
60	27	70
53 5	50	80
64	13	180

* A variation of about 10 per cent of the values is assumed

§ JBI tumour cell concentration 1 5 1 8 × 10⁶/ml

ml and 20 30 × 10⁶ cells/ml for 1 hr at room temperature After centrifugation the cells were resuspended in 4 per cent BSA and painted on cleaned glass slides After fixation in Methanol for 10 minutes the slides were washed in 90 per cent Ethanol over night Furthermore some of the washed cells were suspended in citrate plasma previously centrifuged at high speed to remove particles and after carefully mixing thrombine and calcium were added The clot was immediately fixed in formaldehyde and sections were made of the clot in the rutine histology procedure From here the procedure followed the one used for the smears Ilford K-2 emulsion was used for coating and the slides were exposed for 3-14 days Kodak D 19 developer was used at 16° C for 7 minutes and fixation done in 30 per cent thiosulphate The slides were poststained by Haematoxylin Eosin or in 1 per cent toluidine blue or 0 1 per cent methylen blue

B 2 Results of Autoradiography

The ³H labelled DEAE dextran showed a definite affinity for the tumour cells and the localization of the label seems to indicate that the label is attached to the surface of the cells and does not penetrate to the interior of the cells In the sectioned preparations the label is in most instances localized in ring forms around the cells and there is no tendency to any compartmentalization of the label over the cells Fig 3 About 80 per cent of

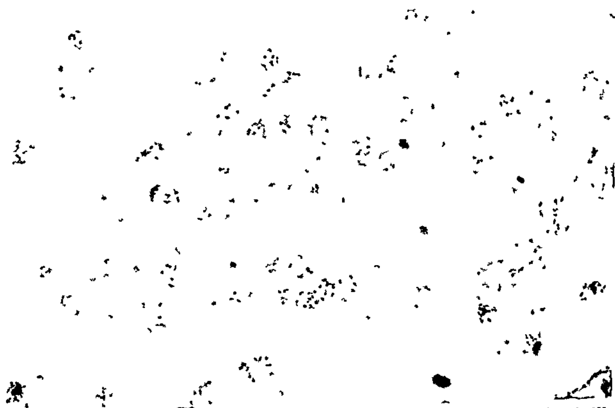
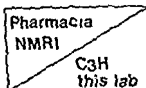


Fig 3 Autoradiographic preparation of tumour cells (JBI ascites plasmocytoma) labelled with ³H DEAE dextran 2000 50

TABLE 2 *Acute Toxicity of Various DEAE Dextrans*

$Mw \times 10^3$ / %Substitution	mg/kg 100	200	400	800	1600	3200	6400	12 800	LD_{50} mg/kg
64/13	0/0	0/0	0/0	0/0	0/0	0/0	0/-	1/-	12.800
60/27	0/0	0/0	0/0	0/0	2/0	3/2	3/3	3/-	2000
188/41	0/0	0/0	0/0	0/2	3/1	3/3	3/-	3/-	1000
535/50	0/0	0/3	0/1	3/3	3/3	3/-	3/-	3/-	550
261/52	0/1	0/2	3/3	3/3	3/-	3/-	3/-	3/-	200
2000/50	1/1	3/3	3/3	3/-	3/-	3/-	3/-	3/-	110

0 no deaths
1 one dead out of 3 mice
2 two dead out of 3 mice
3 all dead



the label could be removed by *neuraminidase* and partly by dextran sulphate Cells previously treated with *neuraminidase* and subsequently incubated with labelled DEAE dextran would also bind the DEAE dextran In these instances it is supposed that the DEAE dextran is bound to other acid groups on the cell surface which have been exposed after removal of the sialic acid component of the mucoproteinlayer (*Larsen & Thorling* 1970)

C 1 Toxicity Determinations in Mice

The acute toxicity was determined as the LD 50 (dead within 24 hrs) by A/B Pharmacia in NMRI mice and repeated in the C3H strain of mice in our lab The appropriate amount of the various preparations of DEAE dextrans were given *i p* in phosphate buffered saline pH 7 4 As shown in Table 2 there is fairly good agreement between the two sets of determinations The LD 50 used here is based on the combined data

In Fig 4 the correlation between the toxicity of the various compounds and their ability to change the surface charge of erythrocytes and JBI ascites tumour cells is shown It appears that for compounds with near the same degree of DEAE substitution the toxicity is linearly correlated to the cell surface affinity in a *double log* system It is seen that the greater the ability is to change the negative surface charge of the cells the greater the toxicity It also appears that the molecular weight of the derivative is decisive for the effect the higher the molecular weight the greater the surface effect and toxicity The two compounds (60/27 and 64/13) with about the same molecular weight

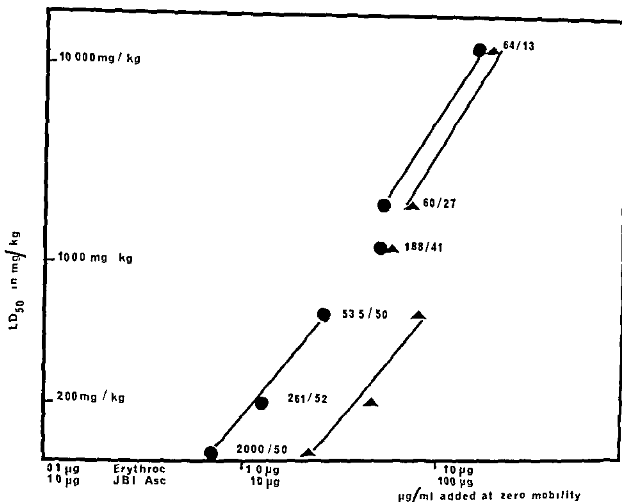


Fig 4 Correlation between toxicity in mice and the surface affinity of the different DEAE dextran derivatives ● results using human erythrocytes ▲ results using JBI ascites tumour cells The surface affinity is expressed as the amount of the derivative necessary to obtain zero mobility in el phoresis

but with different degrees of substitution showed that the toxicity is also dependent upon the degree of substitution the greater the degree of substitution, the greater the surface effect and toxicity

The one compound in the middle of the graft (Fig 4) 188/41 was, as could be expected, somewhere in between what toxicity and surface activity is concerned

C 2 Toxicity in Amoebae

The toxicity of the various DEAE dextrans was tested in Amoebae using the Acantamoebae Castellanii (the amoebae were kindly supplied by Dr *Leif Rasmussen* at the Carlsberg Laboratories, Copenhagen)

The organisms were cultured in Proteose Peptone (Neff and Neff 1964) 5 × 10⁴ cells/ml

The cells were grown in various concentrations of the different DEAE-dextrans, and after 6 days of growth at 31 degrees centigrade, the cells were counted

Smears were prepared for microscopy At low concentrations the cells were almost unaffected, but over a short range of concentrations the growth would cease completely The range is given in Table 3 Microscopy of the cells just surviving the higher concentrations showed marked shrinking

It appears that the relative toxicity of the DEAE-dextrans if analogous to the toxicity in mice, Table 2

Studies on the effect of DEAE-dextran on the surface charge of the acantamoebae have

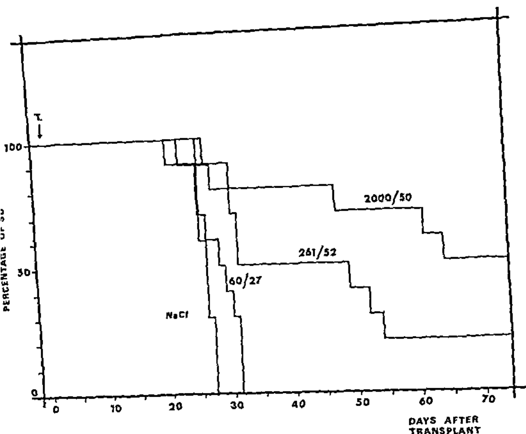


Fig 5 Survival curves for C3H mice transplanted i p with JBI ascites tumour and subsequently given 3 different DEAE derivatives i p after 6 hrs The amount given equals 25 per cent of LD ₀

shown similarities to that of tumour cells although no sialic acid could be demonstrated on the surface of the amoebae

D 1 *Effect on Transplantable Tumours*

The transplantable tumour JBI was used in the ascitic form The tumour was transplanted by giving 1–2 × 10^6 tumour cells i p These cells were obtained from ascites of donor mice transplantated 6–10 days previously The DEAE-dextrans were given also i p 6 hrs after the inoculation of the cells The DEAE dextrans were solved in phosphate buffered saline pH 7 4

Tumour growth was registered by weighing the mice every second day, and the average survival time of the mice was determined

D 2 *Results*

In a preliminary experiment the maximum tolerated dosis of the various compounds was given Already in this first experiment it was evident that the preparation with the lowest degree of substitution (64/13) was without any effect on the development of the ascites tumour Similarly an acetyl amino-ethyl dextran with a molecular weight of 96 000 and 16 per cent substitution was without any effect at all The maximal tolerated dose of the other compounds, however, caused a moderate decrease in the weight of the animals and it was therefore decided to use only half of this dose in the next experiment

The results of this experiment are shown in Fig 5 It appears that the most effective compound was the one with the highest mole

cular weight and a high degree of substitution 2000/50 which was also the compound used in our preliminary experiments already published (*Larsen & Olsen* 1968 *Larsen & Thorling* 1969 *Thorling & Larsen* 1969) The compound 261/50 was slightly less effective and the compound 60/27 was without any significant effect This seems to correlate well with the effect of the compounds on the electric charge of tumour cell surface substantiating our suspision that the effect on the tumour cell surface is essential for the inhibitory effect observed in these experiments

As observed in earlier series of experiments on the *in vivo* inhibition of tumour growth with compound 2000/50 also in this case the growth of the tumour was retarded considerably The survival time was more than doubled and 10–20 per cent of the animals survived with no tumour The animals which did develop tumour usually had a solid tumour rather than the ascitic form

DISCUSSION

There is an increasing amount of evidence supporting the observation of an inhibitory effect of polybases on the growth of transplantable tumours

In recent papers we have investigated in greater detail the effect of the polycation DEAE dextran in the commercially available form with a molecular weight of 2 mil and with di ethyl amino ethyl groups attached to 50 per cent of the glucose molecules as illustrated in Fig 6

The purpose of this present work has been to elucidate the importance of the molecular weight and the charge density of the molecule for its ability to neutralize the negative charge on the cell surfaces and to correlate this to the biological effect of the compound

We have obtained 6 different derivatives of DEAE dextran with molecular weight ranging from 2 mil down to 53 500 and with varying degree of substitution from 52 per cent to 13 per cent Furthermore an acetyl amino dextran has been used

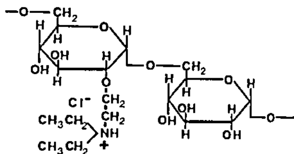


Fig 6 Basic structure of DEAE dextran showing 2 glucose units with one di ethyl amino ethyl group attached to one of the glucose molecules

The binding of these compounds to red cell surfaces has been studied extensively and the results are being published elsewhere (*Larsen & Thorling* 1970)

In this report the binding to tumour cell surfaces has been investigated as demonstrated in Fig 2 and Table 1

The findings in the tumour cell system is analogous to the findings in the red cell system and it is consistently found that the binding is dependent upon the molecular weight as well as on the charge density This is in concert with the observations by *Ryser* (1967) using polyornithine and polylysine He showed that the biological effect was dependent upon the degree of polymerization of the polyamino acids the compounds with the highest molecular weight being the most active Evidently a large molecule with a fairly narrow spacing of the positive groups is decisive for the biological effect indicating that multiple attachment to the cell surface is necessary We found that the charge density was of importance for the effect in a way that the compounds with the highest degree of substitution were the most active

The toxicity of the compounds was investigated in two strains of mice and the LD_{50} determined It appeared that the LD_{50} is correlated to the surface effect as demonstrated in Fig 4 The toxicity was also determined in cultures of the acantamoeba castellani and the relative toxicity of the drugs against this amoeba was the same as in the mouse system the most toxic compound being the one with the strongest binding capacity Table 3

TABLE 3 *Results from 2 Experiments in Which Acantamoebae Castellanii 5 × 10⁴ Cells/Ml Were Cultured for 6 Days in Various Concentrations of Different DEAE dextran Preparations*

DEAE dextran Mw × 10	% substitution	Toxicity in Amoebae conc range growth retard → no growth in µg/ml
2000	50	5–10
261	52	5 10
53	50	10–25
188	41	25–50
60	27	50–100
64	13	>500

The results obtained with the transplantable tumours seem to indicate that a direct interaction between the tumour cell and the polycation is mendatory for the effect Also in this system the effect is dependent upon the ability of the drugs to neutralize the negative surface charge on the cell surface as seen in Fig 5 demonstrating the different survival times in groups of mice treated with 3 different DEAE preparations This effect however does not parallel the toxicity to the mice since all compounds were given in doses corresponding to the same fraction of the LD_{50}

It is believed that the effect on the growth of the transplantable tumours is not a direct toxic killing effect of the drugs on the tumour cells

This is based first on the results of the Lissamine dye exclusion test which shows 75–80 per cent live cells at the time of inoculation of the tumour Secondly it is based on the observation of the reversibility of the inhibition It is possible to obliterate the effect of the incubation with DEAE dextran on the tumour cells by subsequent incubation with Dextran sulphate indicating that no immediately killing of the cells has occurred (*Thorling & Larsen* 1969) also in vivo was this reversion of the inhibition possible for up to 3 days after injection of the DEAE inhibited cells

Thirdly it has been shown that the DEAE inhibited cells will grow in Rtg treated mice almost as well as untreated cells grow in normal mice (*Thorling & Larsen*, to be published)

It is most probable that the effect is a phenomenon taking place in the very surface layer surrounding the cell This assumption is supported by our observations in the autoradiografic assays showing that most of the labelled DEAE dextran can be split off from the labelled cells by neuraminidase and dextran sulphate

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REACTIONS OF THE GUINEA PIG'S SKIN AND ADIPOSE TISSUE TO EXPERIMENTAL FROSTBITE

A Histochemical Study

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Vital and postmortal frostbites of various ages in the skin and adipose tissue of guinea pigs were examined by histochemical methods The microscopic lesions were slight, both in the skin and in the adipose tissue Nuclear changes were seen in vital as well as postmortal frostbites Four hours after the freezing NAD diaphorase and lactate dehydrogenase showed decreasing reactions in the epithelial cells and fibroblasts, and later in fat cells of the frozen area and the reaction had almost entirely disappeared after 24 hours In two to four hours after freezing, diffuse extracellular staining of naphthyl amidase and esterase appeared in the frozen tissue Small fat cell necroses were noticed after a reaction time of four hours Exudation was observed in the sections within one to two hours after vital freezing No morphological signs of damage to vascular walls were seen In blood vessels, closely packed red cells filled the lumen, but real thromboses with fibrin and platelets were rare The inflammatory reaction started in the skin within one hour and in the adipose tissue, within 30 minutes Mononuclear macrophages appeared within four hours in the skin and within two hours in the adipose tissue In frostbites of 16 hours age epidermal blisters filled with fibrinous exudate and inflammatory cells were seen. The blisters were surrounded by a semicircular demarcation zone of polymorphonuclears and macrophages developing within 8–16 hours In the adipose tissue new fibroblasts appeared within four hours after freezing In the skin epithelial proliferation was seen after 36 hours around the blisters The fastest and most reliable vital sign was the post freezing inflammation

The microscopic changes in local congelations most frequently reported in the literature are, capillary wall damage, plasma exudation and thrombosis of small vessels (*Greene* 1943, *Siegmund* 1943 *Kreyberg* 1948, *Friedman* 1946) Ultrastructural injuries which have been demonstrated include changes in the nucleus in the plasma membrane and in cell organell . . .

mation and demarcation of necrosis ensue after these However, the time table followed by the inflammation the histochemical changes that occur, and the distinction between vital and postmortal frostbites, which is important from the forensic medical point of view, have received little attention

In mechanical lesions of the guinea pig's muscle (*Ojala* 1968) and adipose tissue (*Hirvonen* 1968), the main vital reaction was inflammation, which started within thirty minutes after injury In the guinea pig's skin the first vital reaction consisted of accumulation

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of various enzymes such as β glucuronidase, non specific esterase on the edge of incised wounds which was demonstrated in one to two hours (*Rackallio* 1961) In thermal injuries of the guinea pig's skin increase of non-specific esterase was observed *in 45 minutes* and inflammatory reaction in two hours (*Picch 1966*) In the papers cited, the advantages of enzyme histochemical methods in demonstrating vital reactions were emphasized

Postmortal freezing is common in the wintertime if the deceased has met his death out-of-doors Differentiation of vital and postmortal frostbites then possesses practical significance, especially in cases involving suspicion of hypothermal death which is, as a rule, exceedingly difficult to prove

The guinea pig's skin and underlying interscapular adipose tissue which approximate the human skin and subcutaneous fat were considered to be suitable models for studies of the reactions in frostbites Preliminary results indicated that these tissues reacted rapidly to freezing by inflammation (*Hirvonen & Laiho* 1969) The present more comprehensive, series of experiments is largely intended to clarify the usability of histochemistry in examination of frostbite specimens

MATERIAL AND METHODS

Vital experiments were performed on 24 adult guinea pigs under ether anaesthesia Vital frostbites were induced by holding with gentle pressure a copper container filled with dry ice (−70°C) against the shaved skin just above the interscapular fat pad for a period of four minutes This produced simultaneous injury of the skin and of the underlying adipose tissue For purposes of comparison the fat was exposed and directly frozen in 12 cases In these vital experiments the animals were killed by a blow to the head 5 15 and 30 minutes 1 2 4 8 16 24 36 and 48 hours after freezing specimens were excised from skin and adipose tissue immediately after death For postmortal experiments 6 animals were killed in an identical manner and frostbites were caused after cessation of heartbeat Specimens were taken after postmortal reaction times of 2 4 8 12 16 and 24 hours In both types of experiment the area subjected to freezing was encircled using a marker pen in order to insure exact localization From each lesion two specimens were excised including unfrozen tissue from outside the marked area One specimen was fixed in 10 percent neutral formalin for several days and embedded in paraffin the other was immediately chilled in liquid nitrogen and mounted on a cryostat chuck with 10 percent gelatine

Sections (5 μ) from paraffin blocks were stained with Mallory's phosphotungstic acid haematoxylin (PTAH) (*Pearse* 1960), with May Grunwald Giemsa (*Romeis 1948*) for detection of thrombosis with Adams dimethyl aminobenzaldehydenitrite reaction (DMAB) (*Adams* 1957) for detection of exudation and with periodic acid Schiff (PAS) (*McManus* 1960) for observation of exudation and of cells

For enzyme histochemical reactions the chilled specimens were sectioned at 10 μ in unfixed conditions and the sections were mounted on slides.

Enzyme reactions applied

Alkaline phosphatase (AlPase) Reaction was demonstrated by Gomori s metal salt method (*Pearse 1960*), with β glycerophosphate as substrate pH 9 2 Prior to incubation the sections were fixed in cold (4°C) neutral 10 percent formalin for 30 minutes The incubation period was one hour Mounting in glycerol jelly Control 10^{-3} M $NaAs_i$ without substrate

Acid phosphatase (AcPase) Reaction was also demonstrated by Gomori s metal salt method (*Pearse 1960*) pH 5 0 Substrate fixing incubation and mounting were as for alkaline phosphatase Control 10^{-3} M NaF without substrate

Aminopeptidase or naphthylamidase (NA ase) Reaction was demonstrated according to the method of Nachlas et al (*Pearse* 1960) with L-leucyl β naphthylamide as substrate and Fast Blue B as coupling agent pH 6 5 The sections were unfixed Incubation time was 1½ hours Control 10^{-3} M $CuSO_4$ without substrate

α naphthylesterase (Es) Reaction was visualized according to the modification of *Pearse* (1960) with α naphthyl acetate as substrate and Fast Blue B as coupler agent for fat sections The last mentioned salt was found to yield less unspecific staining of the fat ball in the cells The sections were fixed in cold formalin for 30 minutes The reaction mixture was applied to the sections by dropping which resulted in incubation time of about five minutes Control without substrate no inhibitor

NADH diaphorase (NADH d) was visualized according to the method of Scarpelli et al (Pearse 1960) with NADH as substrate Unfixed sections incubation time one hour pH 7 4 Control without substrate no inhibitor

Lactate dehydrogenase (LD) The method of Hess et al was used with natrium lactate as substrate unfixed sect

pH 7 4 Control w

In order to identify the types of cells which were stained in the enzyme reactions some further sections were cut from the same chilled specimens of skin and fat which had served for the enzyme reactions These sections were fixed in 10 per cent formalin over night and stained with Sudan IV followed by haematoxylin counter stain

RESULTS

I Skin

A *Paraffin Sections*

In the epidermis and hair roots elongation of the cell nucleus was usually seen after vital and postmortal lesions The vital lesions of four to eight hours age presented here and there nuclear changes such as pyknosis and karyorrhexis (Fig 1) The same changes were also sometimes seen in postmortal lesions and were thus not usable as a vital sign

In living animals hyperaemia developed in

the sections stained by DMBA and PAS Closely packed red cells appeared in the vessels but real thromboses with fibrin and platelet aggregations (Fig 2) were rarely seen In the walls of the vessels no morphological changes were observed Occasionally small haemorrhages were noted in the dermis

Margination of polymorphonuclear leucocytes in venules began about 15 30 minutes after vital freezing within one hour they had migrated out into the tissue Monocytes appeared within four to eight hours and lymphocytes within 8–16 hours

In the vital lesions of 16 hours age small intraepidermal blisters filled with fibrinous exudate and with inflammatory cells were seen Fig 3) Similar areas containing exudate and inflammatory cells developed subepidermally in some places Usually the subepidermal demarcation zones of inflammatory cells formed at points where the epidermal lesions had been most severe (Fig 4) In the demarcation zones the closely packed polymorphonuclears appeared angular elongated and sometimes broken up into pieces They were usually arranged in rows between collagen fibre bundles An identical phenomenon was also observed in 16 hour-old lesions in other places where the inflammatory cells were closely packed in great numbers

In the postmortally frozen specimens no hyperaemia exudation inflammatory cells epidermal blisters or demarcation lines were observed

B *Enzyme Reactions*

1 *Alkaline and acid phosphatase* – The results of these phosphatase reactions were similar and are therefore reported together These reactions proved to be useful for the demonstration of inflammation Polymorphonuclears displaying strong reactions of phosphatases were distinctly observable among dermal fibroblasts and capillaries Polymorphonuclears were identifiable by their split nuclei which showed no reaction The presence of the polymorphonuclears was further confirmed in the haematoxylin stained frozen sections from every chilled specimen Polymorphonuclears had settled at the endothelium of venules in the lesions of 15 30 minutes age Solitary polymorphonuclears were found subepithelially in the dermis in lesions aged 30 minutes and occurred in groups in lesions which were one hour old Their number had rapidly increased, and in some instances they began to form phosphatase active subepithelial zones after eight hours (Fig 5) These zones of polymorphonuclears were also visible in the paraffin sections of the same time piece (Fig 4) The demonstration of the phosphatases (AlPase in particular) presented the zones more strikingly because the background (hair roots capillaries and fibroblasts) showed less intense reaction Zones which presented numerous projections were rectangular or semicircular Projections consisted of several inflammatory cells lodged in rows along the collagen fibres The mononuclears which appeared in 4–8 hours displayed strong AcPase but weak AlPase reactions

2 *Alpha naphthyl esterase and naphtylamidase* – Results of these enzymes since they presented largely similar results will be reported together These enzymes visualized no

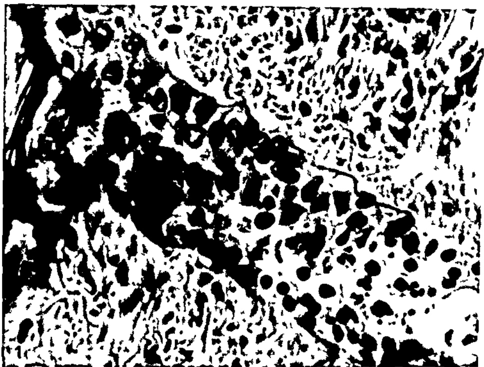




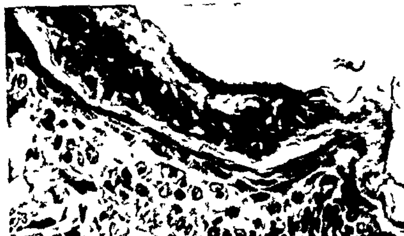
Fig 1 Degenerative nuclear changes including pyknosis and lysis in the hair root and epithelium (on the left) eight hours after freezing PAS × 600

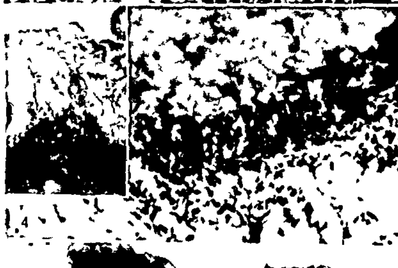
Fig 2 A small thrombus containing fibrin nets platelet aggregates and leucocytes two hours after freezing PTAH × 150

Fig 3 Intra epidermal blister containing leucocytes and fibrinous exudate, 36 hours after freezing The epithelial nuclei show pyknosis PAS × 375

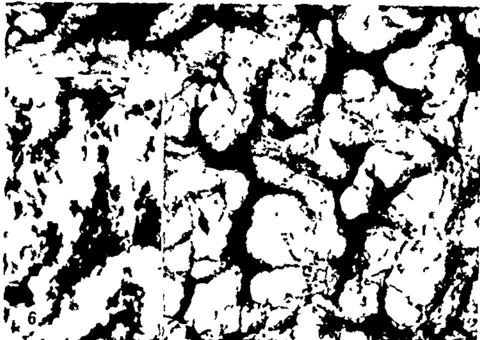
Fig 4 Demarcation zone, 16 hours after freezing The inflammatory cells are packed in rows between the collagen fibres The tissue inside the zone is PAS positive × 375 Insert Esterase reaction in demarcation zone × 375

Fig 5 Another demarcation zone in 16 hour-old lesion Alkaline phosphatase reaction by the metal salt method The inflammatory cells display intense reaction × 375


3


4


5



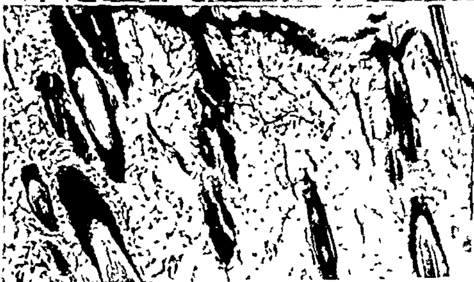


Fig 6 Network-like configurations in the deeper dermis in a 16 hour old lesion Esterase reaction × Insert Frozen section from the same specimen as the esterase reaction showing groups of cells between collagen fibres Haematoxylin × 600

Fig 7 Decline of LDH reaction in epithelium, hair roots and dermal fibroblasts in the frozen area (right, 24 hours after freezing) × 60

early reactions of the skin The number of enzymatically active cells was increased in the dermis, sometimes forming zones, but these were not seen as distinctly as with the phosphatases (Fig 4) Besides the zones there were reticular configurations seen in the dermis after 16 hours, the network consisting of cell groups between collagen fibres as was seen from the frozen sections of the same areas stained with haematoxylin (Fig 6) These cell groups showed distinct reactions of Es and NAase but their cytological character was difficult to evaluate in the enzyme stainings (including the AlPase and AcPase reaction) In addition to the reaction of Es and NAase localized to various cells weak, diffuse general staining of the collagen fibres in the frozen area was seen after two to four hours This

reaction was absent in the intact area and disappeared in sections incubated without substrate or with an inhibitor, as did the cellular reaction

3 *NADH-diaphorase and lactate dehydrogenase* – The normal parts of the skin, including fibroblasts and other mesenchymal cells (hair roots, epithelium, and panniculus carnosus) displayed intense reactions to these enzymes resulting in poor identification of the early inflammatory reaction Weakening of the reactions was observable in the fibroblasts, epithelium, hair roots, and muscle cells in the damaged area after four hours. In the lesions of 24 hours age the epithelium was almost negative (Fig 7) Zones of inflammatory cells displayed an intense NADH diaphorase, but a weaker LHD reaction

In the postmortal frostbites no increase of enzyme positive cells was observable, nor was any decline in the intensity of the oxidative enzymes on the damaged area seen Thus both the increase of enzyme positive cells and the decline of the enzyme reactions in the local skin cells can be regarded as vital signs.

II *Adipose Tissue*

A *Paraffin sections and lipid stainings* – Hyperaemia and minor haemorrhages from capillaries were seen here and there among the fat cells Lesions in the adipose tissue were slight cell necrosis was not remarkable The lesions were mostly concentrated in the periphery of the lobules, where two to three cell rows adjacent to the connective tissue septa were damaged This did not become visible, however until after four hours at which time leucocytes and macrophages had gathered around the fat cells (Fig 8) In contrast, the inflammatory reaction started soon after freezing In the specimens excised as early as five minutes after the polymorphonuclears had begun to lodge in the endothelium of the venules in various regions of the specimen After 15 minutes emigration of the polymorphonuclears into areas outside the vessels had started (Fig 9) and was readily observable after 30 minutes Mononuclear macrophages appeared within two hours first in the septa and then around the damaged fat cells

During 16–24 hours there developed several small focal liponecrotic patches in the fat lobules. In these patches, two to three fat cells had disintegrated and were invaded by polymorphonuclears, macrophages and fibroblasts, or inflammatory cells had gathered to a group between the capillary and the fat cell membrane These necrotic areas did not necessarily appear in connection with larger venules, but the reaction seemed to occur on the capillary level

The freezing lesions were similar in severity and extent in the experiments directed on the exposed fat pad and in those effected through the skin, the inflammatory reaction started equally soon and the lesions were equally slight, in both cases, too, focal necroses were found after 16–24 hours.

B *Enzyme reactions* – The enzyme reactions disappeared with the disintegration of the fat cells. After 16–24 hours there were areas where the fat cells seemed morphologically quite normal, but they had lost the LDH and NAD d reactions, although the capillaries still displayed distinct reactions.

The polymorphonuclears showed intense reactions for AlPase and AcPase and weaker ones for NAase, Es, LDH, and NAD d The polymorphic nuclei were visible as negative, small buttons, confirming the identification Macrophages presented intense reactions for AcPase, NAase, LDH, and NAD d, and weaker ones for AlPase and Es

Since triglycerides only occasionally had escaped from the damaged fat cells, the behavior of the enzyme reactions in phagocytosis could not be followed, but appeared to be more intense in the phagocytes grouped around fat masses than in solitary ones

The first new large fibroblasts were seen in the four to eight hour specimen, becoming most readily visible in the NAase, LDH, and NAD d reactions. The reactions of ALPase, AcPase and Es were much weaker in the fibroblasts Focal necrotic patches were seen in all enzyme stainings, but cells in the patches were not always identifiable because they were

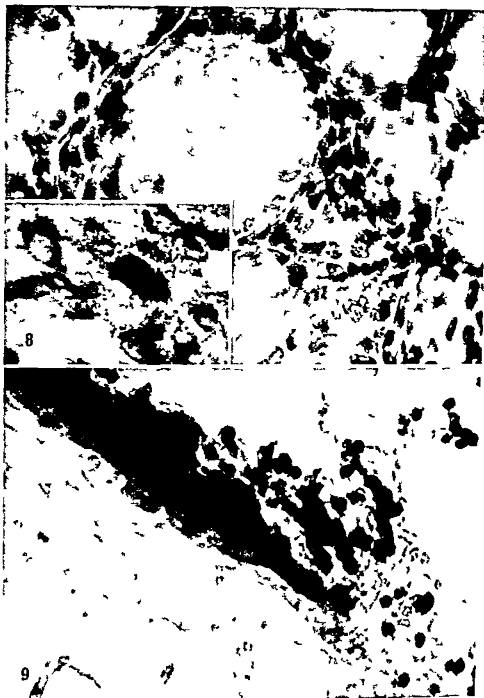


Fig 8 A small focal necrosis in the ad pose tissue 48 hours after the freez ng The fat cells have been surrounded and partly invaded by leucocytes mono nuclear macrophages and fibroblasts The fat cells still contain a triglyceride droplet Sudan/Haematoxylin × 375 Insert NADH d aphorase reaction in macrophages and fibroblasts of the same lesion × 600

Fig 9 Polymorphonuclears with intense alkaline phosphatase reaction in a venule of adipose tissue 15 minutes after freezing A great number of these have lodged in the endothelium some leucocytes have begun to invade the sur roundings Alkaline phosphatase reaction by the metal salt method × 375

so closely packed. A general feature was that the cell types were more easily recognizable in the fat lesions than in the skin. In the lesions induced postmortally, no inflammatory cells, no decline of the reactions of oxidative enzymes in the fat cells, and no other signs of fat cell necrosis were observable.

DISCUSSION

A general finding in the present frostbite experiments was that the *microscopic lesions* were slight both in the skin and in the adipose tissue. Nuclear changes, small blisters, and decline of enzyme reactions were the signs of epithelial damage. Disappearance of enzyme reactions was also observable in the dermal fibroblasts, in the panniculus carnosus, and in fat cells. Lesions in the adipose tissue were small in size, and became visible rather late, not until the polymorphonculears and macrophages had gathered around damaged fat cells. These local fat necroses did not show any preferential localization, but developed at random, and were similar to those reported in the case of human cold panniculitis (*Lowe* 1968). Damage of the capillary wall in frostbite has been observed in earlier studies (*Greene* 1943, *Friedman* 1946), but no clear morphological signs of this were noted in the present study except that occasional small haemorrhages were seen. In blood vessels, closely packed red cells were often observed, but real thromboses with fibrin and platelets were rare. This finding is consistent with earlier observations (*Friedman* 1946, *Essex* & *Quintanilla* 1946). The anoxic influence of the red cell agglutinations may be the same as that of *real thrombi*.

The morphological signs of cell injury seen in the present study were nuclear changes which are similar in nature to those in thermal injuries (*Pioch 1966*), elongation of nuclei, pyknosis, karyorrhexis and karyolysis. Elongation was the most frequent sign. All the changes mentioned were observed both in vital and postmortal frost injuries whereby they are deprived of any importance as vital reactions of frostbite.

After several hours, decline of the reactions of NAD-d and LDH was observable in the epithelium, dermal fibroblasts, muscle cells and fat cells in vital frostbites, and after 24 hours the reactions had almost disappeared. NAD-d is mostly located in the mitochondria and LDH is partly a soluble, partly a mitochondrial enzyme (*Pearse* 1960, *Roodyn* 1967). In electron microscopic studies of freezing and thawing injury of cells, mitochondrial swelling and disruption have been observed. In biochemical analyses made in the same experiments, mitochondrial enzymes displayed decrease of activity (*Trump et al* 1965). The disappearance of the reactions of NAD-d and LDH seen in the present study may reflect mitochondrial injuries. In thermal injuries of the skin decline of oxidative enzyme reactions also occurs, but it is more appreciable and faster, indicating more severe damage of the cells (*Pioch* 1966).

Two to four hours after the freezing, esterase and naphthyl amidase showed diffuse extracellular reaction in the frozen area. In the control sections incubated without substrate or with inhibitor no reaction was seen. Different reasons for this phenomenon may be postulated. According to *Tappel* (1966), in freezing and thawing the lysosomes disrupt and an increase of lysosomal enzyme activity is observed. The appearance of extracellular reactions of NAase and Es may have some connection with the degradation of the cell organelles and with the increased permeability of the injured cell membranes. On the other hand, in EM and biochemical analyses, *Trump et al* (1965) saw no disruptions of lysosomes, and the acid phosphatase activity used as marker enzyme for the lysosomes showed no change. In the present study no histochemical extracellular reaction of AcPase and comparable to those of NAase and Es was noticed. NAase and Es are also present in blood plasma (*Ammon* 1959, *Brown* 1958). One possible explanation of the diffuse reaction is that the enzymes come with exudation fluid. At the time two to four hours after freezing, polymorphonuclears and macrophages containing these enzymes were present

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in the dermis The extracellular reaction may demonstrate the enzymes released from the polymorphonuclears, or may be a methodological diffusion artefact

Despite the slight, visible lesions, the inflammatory reaction started quickly in the skin within one hour at the latest, in the adipose tissue within 30 minutes This speed of reaction is similar to that in skin, muscle and adipose tissue wounds of guinea pigs (*Hirvonen & Ojala* 1968) Slower reactivity of the skin obviously depends on its less profuse vascularity (*Hamberger & Widelitz* 1963) Possibly owing to more severe vascular lesions, the inflammation is more strongly delayed in skin burns in which it starts within two hours (*Pioch* 1966) The epithelial frostbite blisters were surrounded by a semicircular demarcation zone of polymorphonuclears and macrophages, with development of a distinct zone within about 8–16 hours The inflammatory cells were packed in rows between the collagen fibres, producing a striped appearance, whereas in adipose tissue the inflammatory cells occurred in small groups in the focal necroses and connective tissue septa

The enzyme reactions of the inflammatory cells and fibroblasts were more accessible to study in the fat lesions, since in these, identification of cells was easier than in the skin The polymorphonuclears showed intense reactions of AlPase and AcPase, and less intense reactions of NAase, Es, NAD d and LDH Macrophages displayed intense reactions of AcPase, NAase, NAD d and LDH, and weaker ones of AlPase and Es, and the enzyme pattern of the fibroblasts resembled that of macrophages, except of AcPase The demarcation zones in the skin revealed enzyme reactions similar to those of leucocytes and macrophages they were most intensely stained in the case of AlPase AcPase, and NAase Zones stained more strongly in the NAD d reaction than in LDH, since in our experiments the leucocytes showed more intense reaction of the former enzyme

According to the present observations, repair of the lesions began after four hours, in adipose tissue, as indicated by appearance of fibroblasts in the disintegrated areas In the skin, epithelial proliferation around the blisters was seen after 36 hours The speed observed in the fat approximates, or falls slightly short of, that in incised wounds (*Hirvonen* 1968), and the same is true for the skin (*Raekallio* 1961)

With reference to the usability of enzyme histochemistry in investigations of the vital character of frostbites, the results parallel earlier observations made in our laboratory For demonstration of inflammatory cells, enzyme reactions are not absolutely necessary The extracellular reaction of NAase and Es and the decline of NAD d and LDH were vital changes, but they appeared later than the polymorphonuclear leucocytes, which are regarded as a positive sign of vitality, regardless of the fact that one author is of a contrary opinion (*Janezic-Jelacic* 1956) In the staining of leucocytes, the AlPase and AcPase reactions may be useful in that they are more efficient in visualizing polymorphonuclears (especially in the skin) than the other stains employed in the present study

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REVERSION OF THE INHIBITORY EFFECT OF BUSULPHAN ON BONE MARROW CELL PROLIFERATION BY CHLORAMBUCIL

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Serum drawn on the eight and ninth day after the combined administration of busulphan and chlorambucil caused *a distinct increase in the incorporation of* ^{3}H *thymidine* compared with control serum This was taken as evidence that there is in the serum at these points of time a factor that stimulates the proliferation of bone marrow cells and is similar to antichalone in its properties This view was substantiated by the finding that serum taken on the ninth day also increased the uptake of ^{3}H adenine Sera taken on the second and sixth day did not influence the incorporation of ^{3}H thymidine compared with the control serum The stimulating factor was at first weaker after the combined administration of the two cytostatics than after chlorambucil alone but the situation was reversed after the ninth day This was in good accord with the changes in the blood picture The sera now had a distinctly greater stimulating effect on the incorporation of ^{3}H thymidine than the sera taken after busulphan treatment The serum taken after two days was an exception The modes of action of busulphan and its relationship to *chalone are discussed*

The number of leucocytes in the blood of test animals reverts to the normal level more rapidly after the concurrent administration of chlorambucil and busulphan than after busulphan alone (*Elson* 1955 1958) although the amount of alkylating agents is double that after busulphan treatment In addition the mortality of the test animals decreases if chlorambucil is given to rats treated with a busulphan dosage higher than LD $_0$ (*Elson* 1955)

These in a way paradoxical phenomena may be explained as follows Busulphan alone increases the formation of chalone a factor that inhibits the proliferation of bone marrow cells possibly by affecting the system which regulates chalone production but causing relatively slight visible cell damage (*Niskanen* 1967) Chlorambucil on the other hand reacts readily with many chemical groups present in cells (*Warwick* 1963) The consequence is notable cellular damage for instance bizarre mitoses and karyolysis (*Butler & Crathorn* 1958) The combined administration of both alkylating substances would thus prevent the enhancing effect of busulphan on the production of chalone

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and direct cell destruction becomes dominant

A factor by name of antichalone which increases the proliferation of bone marrow cells has been demonstrated in the serum during regeneration after leukapheresis (*Rytömaa & Kiviniemi* 1967, 1968), irradiation (*Cederberg et al* 1967) and the administration of chlorambucil (*Niskanen et al* 1970) The purpose of this work was to find out whether the chalone 'induction by busulphan is influenced by the simultaneous administration of chlorambucil

MATERIAL AND METHODS

White male rats of the Wistar strain were used as test animals Forty five of them were injected intraperitoneally with 12 mg/kg of busulphan and 12 mg/kg of chlorambucil combined in a single dose The substances were dissolved in polyglycol 400 A which was injected without any drug into 45 rats of the control group

The changes in the blood picture were followed at intervals of two days and bone marrow samples were taken at the same time as the serum samples Cellularity was counted from sections of decalcified femurs The differential count was made from smear preparations stained with May-Grunwald Giemsa

Sera were collected 2 5 6 8, and 9 days after the administration of the cytostatics Blood was drawn from the rats by cardiac puncture under light ether anaesthesia The blood was centrifuged and the serum was kept in a freezer at —20° C until use The sera used in the tests were pooled They were tested by the short term tissue culture method described in detail earlier (*Rytomaa & Kiviniemi* 1967 *Niskanen* 1967 *Rytomaa* 1969)

^{3}H thymidine (sp ac. 3 0 Ci/mM) was used as the indicator of DNA synthesis to elicit the proliferative activity of the cells in tissue cultures RNA synthesis was indicated in these tests by the incorporation of ^{3}H adenine (sp ac 2 36 Ci/mM) in the cells The amount *of radioactive substance in the* cells was measured in a Packard Tri-Carb liquid scintillation counter The number of cells adhering to the [illegible]

[illegible] s obviated the effect on the results of possible detachment of cells

Part of the cover glasses analysed as described were washed in series of solutions of dioxan and water to remove all scintillator liquid from the samples The preparations were then mounted face up on microscope slides After this autoradiograms were prepared by the Kodak AR 10 stripping film technique The cells were stained with Mayer's haematoxylin solution. The autoradiograms were analysed by counting the proportion of labelled cells from code numbered preparations

The results were analysed at the Computer Centre of the University of Helsinki in an IBM 1620 digital computer The observations for the different test series were not comparable as such because the cell and isotope amounts varied in addition to which the incubation times were different It was therefore necessary to re-scale individual values with the aid of the formula $\frac{x_i \; x_c}{x_c}$ in which x_i is the value to be converted and x_c the mean value of the control group of the corresponding test series Each experimental series was tested within a time group by the *t* test Results for sera taken at the same time were pooled and tested for statistical significance by the *t* test and F test The result of the two tests was naturally the same because of standardization to ensure that only one variable was involved The *t* test was applied to compare the results with those obtained in earlier studies (*Niskanen* 1967, *Niskanen et al* 1970)

In some instances Wilcoxon's 'nonparametric' method was applied

RESULTS

The changes in the cell counts of the blood and bone marrow were in full agreement with the results reported in earlier studies (*Elson* 1955, 1958), for which reason no detailed values are given here This part of the study was conducted in fact solely for purposes of control

Serum taken two days after combined busulphan and chlorambucil treatment did not affect the proliferation of normal bone marrow cells in tissue cultures (Table 1) The blood and bone marrow cell counts were both close to the minimum (*Fig 1*)

The next serum to be tested was taken at five days when changes suggestive of incipient regeneration were seen in the bone marrow and blood Serum stimulated the incorporation of ^{3}H thymidine into bone marrow cells in tissue cultures (Fig 1) The effect, however, was not a full 10 per cent The result was significant at the level $p < 0 01$ (Table 1)

Serum drawn on the sixth day revealed no definite changes compared with the control

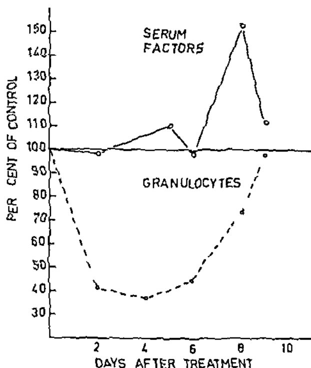


Fig 1 Relative alterations after concurrent administration of busulphan and chlorambucil in the number of blood granulocytes and serum factors controlling cellular proliferation in bone marrow

The bone marrow was hypoplastic at this time and the blood granulocyte count was only slightly higher than the minimum value (Fig 1)

No distinct evidence of enhanced ^{3}H thymidine uptake was seen until the serum taken on the eight day The result was about 90 per cent in the first test series a considerably higher figure than has usually been obtained with this method It is probable that other factors than just the effect of the serum of the test group affected the result but as no rational explanation was found the result was accepted In a repeat test the stimulation was 15 per cent which is much closer to the usual values (Table 1) In the autoradiograms from the same cover glasses the number of cells displaying isotope incorporation exceeded that in the respective controls by 21 7 per cent (Fig 2) The difference is statistically significant at a level of $p<0\ 01$

The blood granulocyte count was already nearly normal on the ninth day after the treatment (Fig 1) and cellularity in the bone marrow was also at almost the original level Serum taken at the same time showed an 11 4 per cent increase in the uptake of ^{3}H thymidine in the bone marrow cells The effect was not pronounced but it was statistically highly significant ($p<0\ 001$) The incorporation of ^{3}H adenine indicating RNA synthesis increased by 21 6 per cent which was also statistically significant at the level $p<0\ 001$ (Table 1)

The erythrocyte count also declined after the treatment, but at a much slower rate than the leucocyte count At 10 days the number of erythrocytes in the blood was half of the normal count

DISCUSSION

It can be concluded from the results that the changes in cellularity that occur in the bone marrow and blood after combined busulphan and chlorambucil treatment are based on two separate mechanisms The initial rapid decrease was obviously due to the direct destruction by the cytostatics of cells capable of mitosis (*Elson* 1955 1958) On the other

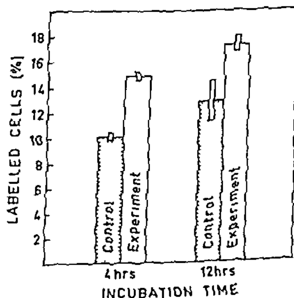


Fig 2 Per cent of labelled normal bone marrow cells incubated *in vitro* in the presence of test and control sera (mean + S D N = 6 $P < 0\ 001$) The test serum was obtained 8 days after combined busulphan and chlorambucil treatment

TABLE 1 *Effect of Sera Obtained after Combined Busulphan and Chlorambucil Treatment on ^{3}H Thymidine and ^{3}H Adenine Uptake in Normal Bone Marrow Cells* In Vitro

Type of sera Number of experiment/incubation time (hrs)	Type of radioactive precursor activity (counts/100 sec) Control Mean±S E	Exp Mean±S E	Difference (per cent of control)	t test value	Degrees of freedom	Significance
2nd day sera	^{3}H thymidine					
I/4	1480± 57	1258± 44	−15.1	3.36	13	$p < 0.01$
I/15	1530± 70	1544± 93	+ 0.9	0.13	13	
II/4	1146± 47	1178± 46	+ 2.8	0.53	13	
II/12	1188± 52	1229± 67	+ 3.5	0.52	13	
		Total	− 2.0	0.77	54	
5th day sera						
I/4	1809± 83	2182±139	+20.5	2.42	19	$p < 0.05$
II/4	3272±277	3778±206	+15.5	1.54	19	
II/22	3833±227	3200±182	−16.5	2.29	19	$p < 0.05$
III/24	994± 52	964± 73	− 3.0	0.35	23	
IV/3	342± 37	432± 31	+26.3	2.00	13	$p < 0.1$
V/5	1650± 93	2018±128	+22.3	2.45	19	$p < 0.025$
V/22	2885±169	2988±161	+ 3.6	0.46	19	
		Total	+ 9.8	2.82	134	$p < 0.01$
6th day sera						
I/4	770± 84	845± 40	+ 9.7	0.88	13	
I/12	1308± 73	1205± 80	− 7.8	1.03	13	
II/3	386± 28	379± 11	− 1.8	0.23	13	
II/12	678± 46	641± 42	− 5.5	0.64	13	
		Total	− 1.4	0.50	54	
8th day sera						
I/4	1463± 76	2380± 50	+82.0	10.87	13	$p < 0.001$
I/12	1134± 50	2234± 93	+97.0	11.24	13	$p < 0.001$
II 4	1520± 88	1737± 54	+14.3	2.28	13	$p < 0.05$
II/12	1208± 71	1394± 50	+15.4	2.30	13	$p < 0.05$
		Total	+52.2	6.70	54	$p < 0.001$
9th day sera						
I/4	4045±242	4582±374	+13.3	1.28	17	
I/23	4478±200	5553±316	+24.0	3.05	17	$p < 0.01$
II/3	1451± 72	1349± 77	− 7.0	1.02	19	
II/22	1760±142	1970±107	+11.8	1.25	19	
III/4	1852± 93	2000± 98	+ 8.0	1.19	13	
III/12	2339±191	3087±199	+32.0	2.93	13	$p < 0.02$
IV 4	4419±131	4705±266	+ 6.5	1.04	13	
IV/14	2845±134	3781± 75	+32.9	6.60	13	$p < 0.001$
V 5	1441± 68	1629± 37	+13.0	2.63	13	$p < 0.025$
V/15	1295± 43	1320± 55	+ 1.9	0.39	13	
		Total	+11.4	5.82	158	$p < 0.001$
9th day sera	^{3}H adenine					
I/4	1908±169	1888±161	+ 1.0	0.15	13	
I 12	2887±172	3889±138	+34.7	4.91	13	$p < 0.001$
II/4	1150± 41	1137± 49	− 1.1	0.22	13	
II/12	1878± 66	2851±211	+51.8	4.74	13	$p < 0.001$
		Total	+21.6	3.88	54	$p < 0.001$

TABLE 2 *Difference in the Effects of Sera Obtained after Busulphan, Chlorambucil and Concurrent Busulphan and Chlorambucil Treatment*

	Busulphan— Chlorambucil	Busulphan— Chlorambucil + Busulphan	Chlorambucil— Chlorambucil + Busulphan
2nd day sera (^{3}H thymidine)	D = 60 9 % t test value = 7 80 d f = 37 p < 0 001	D = 61 9 % t test value = 8 33 d f = 37 p < 0 001	D = 1 0 % t test value = 1 00
5th day sera			D = 9 3 % t test value = 0 53 d f = 94
6th day sera	D = 42 3 % t test value = 6 32 d f = 38 p < 0 001	D = 20 4 % t test value = 3 57 d f = 38 p < 0 001	D = 18 9 % t test value = 3 39 d f = 48 p < 0 05
8th day sera	D = 71 0 % t test value = 8 96 d f = 38 p < 0 001	D = 69 4 % t test value = 8 68 d f = 38 p < 0 001	D = 1 6 % t test value = 0 48 d f = 48
9th day sera	D = 26 0 % t test value = 6 44 d f = 160 p < 0 001	D = 31 7 % t test value = 7 68 d f = 160 p < 0 001	D = 5 7 % t test value = 2 66 d f = 140 p < 0 01

The results in this table give the differences in the action of sera taken after the administration of busulphan, chlorambucil and the two alkylating agents combined The values could be compared after they had all been adjusted to the same scale in which the means for the control groups were at the O level D = the relative value of the difference of the means for the test groups t value = value given by Student's t test d f = degrees of freedom p = level of statistical significance The table reveals only the magnitude but not the direction of effect of the serum The direction is presented in Fig 3

hand, a proliferation-stimulating factor was demonstrable in the serum during regeneration

In an earlier study (*Niskanen* 1967) we found that serum taken two days after the administration of busulphan increases the uptake of ^{3}H-thymidine (but not cell proliferation) *in vitro* The same phenomenon is produced by busulphan itself, which suggests that the drug is still present in the serum two days after its administration Serum drawn two days after the administration of chlorambucil did not influence the incorporation of ^{3}H-thymidine *in vitro* (*Niskanen et al* 1970) As the situation is the same when both cytostatics are given together, it is obvious that in this case no busulphan remained in the serum after two days A possible reason is that chlorambucil diminishes inter cellular adhesive attraction (*Froese et al* 1969) the consequence of which might be easier penetration of busulphan into tissue and, thus, accelerated disappearance from the serum

After the administration of chlorambucil alone the leucocyte count in the blood begins to rise slightly more rapidly than when it is given in combination with busulphan (*Elson* 1955, 1958) A comparison of the results of the present study with those published earlier (*Niskanen et al* 1970) revealed that the appearance in the blood of a factor stimulating the proliferation of bone marrow cells accords well with the changes that occur in the blood picture in both cases Fig 3 and Table 2 show that the effect of the stimulating factor after the combined administration

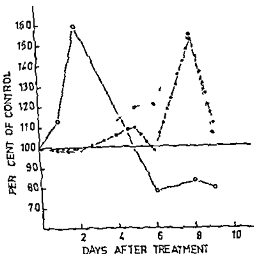


Fig 3 Relative alterations in the effects of sera taken after busulphan (o——o) chlorambucil (o- -o) and concurrent busulphan and chlorambucil (o-o-o-o) administration on ³H thymidine incorporation in normal bone marrow cells *in vitro*

of two cytostatics was weaker initially than after chlorambucil alone, but that the stimulating effect was possibly maintained a little longer Sera drawn at the corresponding times after busulphan alone inhibited the proliferation of bone marrow cells *in vitro* which was attributed to chalone (*Niskanen* 1967) The reaction differed statistically from the stimulating effect established in another study (*Niskanen et al* 1970) and here This can be seen from Table 2

The results now obtained confirm the view that busulphan causes a unique change in the production of regulating factors (*Niskanen* 1967) If the long term inhibition caused by busulphan were due to the formation of a toxic factor and not to chalone, it would be difficult to understand how the administration of another cytostatic chlorambucil to the same animals would make the reaction stimulative

Busulphan acts primarily on immature dividing cells of the granulocyte series which first decrease in number followed by a subsequent decrease in the number of mature cells (*Niskanen* 1967) The changes under the influence of chalone take place in the same order (*Rytömaa & Kiviniemi* 1967, 1968) If the animals are given chlorambucil as well, the blood granulocyte count declines fairly fast (*Elson* 1955, 1958) Temporally it is not possible that the rapid decrease is caused by the inhibition of cells induced by chalone production and thus it is obviously a direct destructive action of cytostatics that is in question Chalone is probably not formed in the presence of chlorambucil, though there is busulphan in the organism The assumed reason for this is that the additional action of chlorambucil caused such great damage in the cells that the mechanism responsible for chalone production is destroyed

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ENTERIC INFECTION WITH *YERSINIA ENTEROCOLITICA*

Large Pyroninophilic Cell Reaction in Mesenteric Lymph Nodes Associated with Early Production of Specific Antibodies

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Enteric infection with *Yersinia enterocolitica* is often associated with small focal non specific ulcerations in the appendix and sometimes with basophilic stem cell hyperplasia or proliferation of large pyroninophilic cells in mesenteric lymph nodes This proliferation correlates comparatively well with an early antibody response The clinicopathological and immunological aspects of the findings are discussed

Yersinia enterocolitica (9), formerly *Pasteurella X*, has been stressed as a cause of enteric infections and mesenteric lymphadenitis by several authors (4, 12, 22, 23 27, 38), but in those few studies in which the morphology is mentioned (4, 23, 38) the interest seems to have been focused on details other than those to be described

Histological examination of a mesenteric lymph node from a child with signs of acute appendicitis (case 4) showed capsular and trabecular inflammation and proliferation, in the cortical parts of the pulp, of large pyroninophilic, slightly vacuolated cells with large nucleoli We consider them to correspond to the basophilic stem cells (Basophile Stammzellen) of *Lennert* (1961) and synonymous with large pyroninophilic cells (24) Mitotic figures were numerous The alterations differed from those in the reticulocytic abscess forming lymphadenitis (19,20) caused by *Yersinia pseudotuberculosis* (14) by the absence of typical abscesses, and also from the diffuse hyperplasia commonly found in so called non specific mesenteric lymphadenitis (17) *Yersinia enterocolitica* (*Y e*) was isolated from the above node Based on a similar lymph node morphology, infection with this micro organism was suspected and later verified bacteriologically in three further cases (cases 6, 7 and 8), our cases of infection with *Y e* have not all had a prominent proliferation of large pyroninophilic cells

Received 3 viii 70

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LABORATORY METHODS

The histological specimens were fixed in 10 per cent phosphate buffered neutral formalin generally The appendices were fixed unopened All slides were stained at least with haematoxylin eosin and methyl green pyronine

All histological data were recorded and tabulated before most of the serological examinations had been carried out and before they were known to

the morphologist The findings were graded from — to + + + so that a ± alteration was rather questionable, not differing significantly from those seen in *e g* almost any reactive lymph node We have tried to use the terminology of *Lennert* (1961) and *Mori* & *Lennert* (1969), to make it short we have often omitted the original terms used by other authors

The bacteriological cultures were made by the methods of *Niléhn* & *Sjostrom* (1967) with slight modifications The identification of the *Yersinia* strains was based on typical cultural and bio chemical characteristics (15, 26) and on agglutination in type specific O factor antisera (36)

Yersinia agglutinins were determined as described earlier (1) All sera were tested for agglutinins against two *Y e* serotypes (Strains "Winblad and M Y 79, referred to here as types III and IX, respectively) and against *Y pseudotuberculosis* type I, and in selected cases against other serotypes Titres given in this paper were obtained with the O antigen of *Y e* type III and the OH-antigen of *Y e* type IX (1)

The immunofluorescence was carried out by fixing formalin killed and washed *Yersinia* bacteria on slides with absolute ethanol They were incubated with patient sera, starting at dilution 1 5 in a moist chamber for 30 minutes, and washed twice in 0 01 M phosphate buffered saline, pH 7 2 Subsequent treatment with FITC-conjugated anti human globulin and anti human IgM, IgG, IgA and IgD was carried out as above

The 2 mercaptoethanol sensitivity of the agglutinins was tested according to *Uhr* & *Finkelstein* (1963)

Immunoglobulin levels were determined by the radial immunodiffusion method

OBSERVATIONS

1 *Material*

All our microscopically studied cases of enteric infection with *Y e* during the three year period ending in June 1969 will be described The material from which the series of infections with *Y e* was collected is shown in Table 1 During this period cases with questionable or incipient inflammatory alterations had been coded as incipient appen

TABLE 1 *Histologically Studied Appendices and Mesenteric Lymph Nodes The Appendices Have Been Grouped According to the Coded First Microscopic Diagnosis, All the Lymph Nodes Have Been Re Examined*

	Total number	Examined for Yersinia	Positive for	
			Y enteroc	Y pseudotub
Appendices				
Normal, no diagn alt (23 faecaliths)	177	42	–	1 (Type III)
Focal appendicitis (1 faecalith)	14	6	5	–
Incipient appendicitis (2 faecaliths)	28	6	2	2 (Type I)
Acute or gangraenous appendicitis (53 faecaliths)	145	8	–	–
Other mostly inflamm cond (13 faecaliths)	126	15	–	–
All cases except neoplasms (92 faecaliths)	490	77	7	3
Mesenteric lymph nodes				
Normal	4	3	–	–
Large pyroninoph cell reaction	10	7	4	–
D o with reticulocytic abscesses	3	2	–	2 (Type I)
Diffuse hyperplasia	22	13	–	–
Other infl & hyperplasias	41	24	2	1 (Type III)
All cases except neoplasms	80	49	6	3

dicitis In retrospect it was found that the condition in the first report often had been described as focal in such incipient cases as later were found to be associated with Y e

The cases with focal alterations were collected into a separate group All our cases presenting evidence of infection with Y e were found in these two groups most of them in the focal appendicitis group Rather few of our cases of established acute or gangraenous appendicitis often associated with macroscopical or microscopical faecaliths had been examined for Yersinia Not all appendices have been re examined and it is quite possible that some cases coded as normal may have had some small focal ulcerations

All the lymph nodes were re examined A large pyroninophilic cell reaction was prominent in 10 cases seven of these had been examined for Yersinia with a positive result in 4 cases Bacterial or serological examination had revealed Y *pseudotuberculosis* type I in two out of three in which in addition to large pyroninophils there were reticulocytic abscesses In addition *to three of the* four cases with a pyroninophilic *reaction the* morphologist had suggested infection with Y e in one case that was caused by the less common type III of *Y pseudotuberculosis* None of the 13 cases examined for *Yersinia* and diagnosed as diffuse hyperplasia (17) showed evidence of infection with this group of bacteria

2 *Clinical Haematological and Bacteriological Data*

The main data are shown in Table 2 In case 1 the symptoms of appendicitis had been preceded by diarrhoea for three days in case 6 for a week According to the surgical report the appendix was normal in most *cases* The terminal ileum was *mentioned* in two cases only *thus* we cannot state anything about the frequency of acute terminal ileitis which has been reported to occur in infection with Y e (38)

Differential white blood cell counts had been made in cases 4 and 6 only, in addition to a shift to the left cells said to be plasma cells had been found in both cases in numbers of 3 and 7 per 200 leucocytes respectively

In every case Y e was isolated from at least one specimen It was found in the lymph node proper only in cases in which proliferation of large pyroninophils was prominent (Tables 3 and 4) In case 8 infection with two different *serotypes of Y e* was evident Agglutinins against the corresponding serotypes were detected in every case

3 *Alterations in the Appendix*

Gross examination of the *fixed* appendices revealed often a few mucosal haemorrhages and slightly dilated serosal vessels In all but *case 5 the microscopical* examination showed *small focal* ulcerations (Fig 1) associated with neutrophils and bacteria focally distributed in the lumen (Table 3) The ulcerations were sometimes connected with the upper parts of the germinal centres The number of mitoses and tingible bodies (activity') in the germinal centres *varied* microabscesses of the kind *described by others* (38) were seen only in case 2 Signs of incipient desquamation of the mucosa were present in case 3 only The luminal parts of the lamina propria contained always an apparently normal number of mature *plasma* cells Occasionally the deeper parts of the interfollicular pulp contained large pyroninophils and mitotic figures Serosal and muscular layer *inflammation* was present in one case only

4 *Mesenteric Lymph Node Morphology*

The microscopical findings are summarized in Table 4

Cases 4 6 7 and 8 with proliferation of large pyroninophilic cells The capsule, the trabeculae and the perivascular areas were swollen oedematous sometimes metachromatic and infiltrated with some neutrophils eosinophils and plasma cells in a few cases the lumina of larger blood vessels were nar

the morphologist The findings were graded from — to + + + so that a ± alteration was rather questionable, not differing significantly from those seen in *e g* almost any reactive lymph node We have tried to use the terminology of *Lennert* (1961) and *Mori* & *Lennert* (1969), to make it short we have often omitted the original terms used by other authors

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All cases except neoplasms	80	49	6	3

in *Patients with* Yersinia enterocolitica *Infection*

	5	6	7	8
	10 ♂ 1 day	5 ♀ 1 day	43 ♀ 3 days	14 ♂ 2 days
intermitt	–	7 days diarrhoea	–	–
	15 900 39 5 C	25 100 38 6 C	9 000 37 5 C	16 000 38 7 C
(	Normal	Swollen	Normal Swollen	Normal ·
reddish	Small, firm	Large, firm	Enlarged	Enlarged
	III	III	III	III and IX
	+	+		+ +
	—	+		+ +
	+	+	+	+ +



s of the appendix in case 2 (left) and case 5 (right) stained with methyl green pyronine and H & E respectively × 170

TABLE 2 *Clinical Haematological and Bacteri*

Case	(1)	2	(3)
Age (years) sex	12 ♂	13 ♀	15 ♂
Symptoms of appendicitis	1 day	3 days	3 days
Prodromal symptoms	3 days diarrhoea	4 weeks intermitt pain	–
Leucocytes/mm³ preoperatively	12 700	9 000	11 000
Rectal temp preoperatively	38 2 C	38 0 C	38 2 C
Gross findings at laparotomy			
Appendix	Normal	Normal	Normal
Terminal ileum			Swollen
Mesent lymph nodes	Slightly enlarged	Enlarged	Not enlarged
Bacterial culture			
Type of *Y enterocolitica*	III	III	III
Appendix		+	
Mesenteric lymph node	+	—	
Faeces		+	+

Lymph nodes were not examined microscopically in the cases given in brackets

rowed The size of the follicles and the germinal centres varied to a large extent (Fig 2) sometimes the follicles were very small consisting of only a few subcapsular islands of small lymphocytes (Fig 2 left) very few germinal centres being found In case 6 the germinal centres contained a fair number of neutrophils

The most prominent alterations were seen in the cortical parts of the pulp at the sides of and below the follicles If germinal centres were present the pulp alteration was separated from these by a narrow lymphocytic cuff The cortical pulp was enlarged and contained numerous enlarged cells and mitotic figures (Figs 3 and 4) The nuclei of these large cells were round or indented with a slight marginal condensation of chro

TABLE 3 *Morphological Alterations in the Appendix*

Case	1	2	3	4	5	6
Distrib of neutrophils in lumen	general?	focal	general	focal	focal	
No of ulcerations	3	6	7	5	—	2
Germinal centres						
Activity	+ +	+	+ +	+ +	+ +	+
Neutrophils	—	+	—	—	—	—
Interfollicular pulp						
Large pyroninophils	+ +	+ +	+ ?	+ foc	+	+ +
Mitoses	+ +	+	±	+ foc	—	+
Muscular and serosal inflamm	—	—	—	few neutropl	—	—

In case 6 the appendix had been opened before fixation the appendix in case 7 was not studied microscopically



Fig 2 Sections of mesenteric lymph nodes in case 7 (left) and case 6 (right) stained with H & E × 10

matin and one or several huge nucleoli often located close to the nuclear membrane (Fig 4) The cytoplasm was usually vacuolated and clearly though not very strongly pyroninophilic The cells seemed often to be intimately connected with small vessels with large endothelial cells perhaps corresponding to postcapillary venules (11) Transitions between these large cells regarded to correspond to *Lennert's* (1961) basophilic stem cells synonymous with large pyroninophilic cells (24) and smaller more strongly pyroninophilic cells seemed to be present but mature plasma cells were sparse In most cases it was difficult to distinguish cortical pulp from medullary cords and sometimes the cellular proliferation may have affected the deeper parts of the nodes in addition to the cortical pulp In case 8 only a part of the cortical pulp seemed affected by the stem cell hyperplasia

Usually, the marginal sinuses could be recognized easily and they often contained comparatively large pyroninophils and mitotic figures (Fig 5) Usually it was difficult to distinguish intermediary sinuses, if properly identified they often contained differently sized, large and sometimes pyroninophilic lymphoid cells In case 6 there was an immature sinus histiocytosis (17) resembling that seen in *e g* toxoplasmosis A typical mature sinus histiocytosis, if present at all was weak There were scattered neutrophils and eosinophils, the number of mast cells seemed low Neither epitheloid cell granulomas nor any reticulocytic (histiocytic) abscesses were demonstrable In case 6, the only one in which large efferent lymph vessels

TABLE 4 *Mesenteric Lymph Node Alterations in Our Cases, and the 'Mean' Alterations in Some Entities to be Remembered in Examinations of Mesenteric Lymph Nodes and in Piringer's Lymphadenitis as Seen in Cases of e g Toxoplasmosis*

Case	2	4	5	6	7	8	Diffuse hyperplasia	Pseudo tuberc lymph adenitis	Piringer's lymph adenitis
Capsular and trabec inflamm	±	+ +	—	+ + +	+ +	+	—	+ +	+ +
Follicle size	+	+ +	±	+ +	±	+ +	Var	Var	+ + +
Germ centre activity	+	+ +	—	+ +	±	+ +	+	Var	+ + +
Cortical pulp									
Size	+	+ +	+	+ +	+ + +	+ +	Var	+ +	Var
Mitoses	±	+	—	+ + +	+ + +	+ +	Var	Var	Var
Large pyroninophils	+	+ +	±	+ + +	+ + +	+ +	+	+ +	Var
Medium sized pyroninophils	+	+ +	+	+ +	+ +	+ +			
Sinuses									
Mature histiocytosis	+	+	+ +	—	—	+ foc	—	Var	Var
Immature histiocytosis	—	—	—	+	—	—	—	±	+ + +
Small lymphoid cells	+	+	+	+	+	+ +	+ + +	Var	Var
Large lymphoid cells	—	+	±	+ + +	+ + +	+ + foc	±	Var	Var
Reticulocytic abscesses	—	—	—	—	—	—	—	+ + +	+ ?
Small granulomas	—	—	—	—	—	—	—	+ ?	+ + +

There was no clear correlation between the serological findings and the alterations in the appendix

Case 4 seems to be one of hypogammaglobulinaemia A and the IgA levels were fairly low also in cases 7 and 8 but there was no significant correlation between these levels and the large pyroninophilic cell reaction in the lymph nodes

DISCUSSION AND CONCLUSIONS

Large Pyroninophilic Cell Reaction in Human Pathology

To our knowledge *Lennert* (1961) is the only pathologist who at least at a text or handbook level has described a clearly defined entity observed in man and apparently corresponding to the proliferation of the large pyroninophils seen in the lymph nodes of four of our cases *Lennert* called these cells basophilic stem cells (basophile Stammzellen) and the process stem cell hyperplasia (Stammzellen Hyperplasie) To avoid confusion with the stem cells referred to by immunologists (3) we have used the term large pyroninophilic cell which according to *Mori & Lennert* (1969) is a synonym *Lennert* stated that these cells often are found in the acute stage of non specific lymphadenitis and that stem cell hyperplasia later turns into follicular hyperplasia

A prominent stem cell hyperplasia according to *Lennert* (1961) often is seen in cases of reticulocytic abscess-forming lymphadenitis (*e g* cat scratch disease lymphadenitis due to *Y pseudotuberculosis*) in

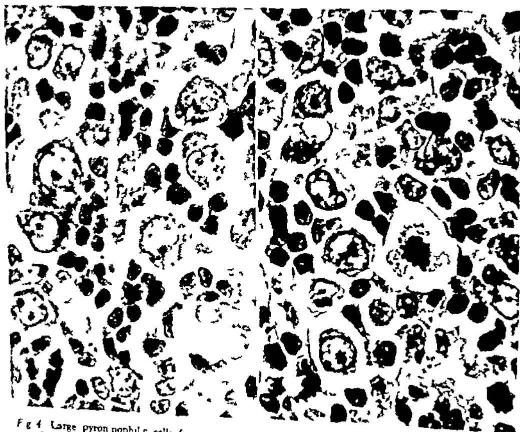


Fig 4 Large pyroninophilic cells from case 6 left) stained with methyl green pyronine and from case 7 right) stained with H & E × 1300



Fig 3 The large pyroninophilic cell reaction in case 6 demonstrated with methyl green pyronine staining × 400

could be identified, these contained large and often pyroninophilic cells and some mitoses (Fig 5)

Cases 2 and 5 The alterations in these cases were rather unimpressive, although at re-examination a few large pyroninophilic cells were found Mitotic figures were sparse

5 *Correlation between the Morphology and the Serology*

The main results are shown in Table 5 High agglutinin titres against *Y e* in the first sera obtained were found in those cases only which also clearly showed proliferation of large pyroninophilic cells in the mesenteric lymph nodes According to the Friedman rank test there was an almost significant correlation between the degree of this proliferation and the *Y e* agglutinins ($P<0\ 05$) in the first sera In cases 4, 7, and 8 the *Y e* agglutinin titres dropped to 1 40 or less in the first serum after treatment with 2 mercaptoethanol, whereas in case 6 the titre dropped three twofold dilution steps only These results are substantiated in part by the indirect immunofluorescence tests, in which all but cases 6 and 8 showed an initial preponderance of IgM antibodies although with a simultaneous appearance of IgG antibodies In case 6, the symptoms of appendicitis had been preceded by diarrhoea for a week In case 8 two serotypes had been *found, in Table 5, only titres against type* III are given IgA antibodies were found in some cases, IgD antibodies were not detected There was an almost significant correlation between the IgM levels in the first sera and the degree of large pyroninophilic cell reaction ($P<0\ 02$) in the lymph nodes, but none between the first agglutinin titres or IgM levels and the number of germinal centre mitoses and tingible bodies ("activity')

Germinal Centre Activity in the Mesenteric Lymph Nodes at Laparotomy (Day 1) and the Serum Immunoglobulin Levels (mg/100 ml) Per and Postoperatively

5				6		7				8			
1	6	20	48	1	16	3	6	22	50	1	6	20	48
+ –				+ + + + +		+ + + ± } on day 1				+ + + +			
<20	320	2560	320	10240	640	2560	5120	320	–	640	2560	640	40
	80 5 –	80 320 20 –	80 320 – –	80 320 320 –	5 320 20 –	80 20 80 –	20 20 80 –	80 80 20 –	5 20 – –	80 320 5 –	320 320 20 –	20 320 – –	5 80 – –
56 940 165	64 1270 220	76 1570 187	38 1250 174	150 1560 220	180 2080 185	171 1500 135	108 1630 146	124 1730 124	156 1740 140	139 930 130	110 1200 160	150 1250 155	

Our tabulated data do not, contrary to expectations show any clear relationship between the alterations in the appendix and those in the mesenteric lymph nodes, nor between the alterations in the appendix and the serological findings Larger series should be studied with regard to a possible relationship between IgA levels and the mesenteric lymphadenitis It should be noted that case 4 one of hypogammaglobulinaemia A did not seem to have subnormal numbers of plasma cells or germinal centres in the appendix It is not known whether the lymph node reaction is related to the arthritis (1) and erythema nodosum (1 37) described in association with Y e infection

Immunological Significance of the Large Pyroninophilic Cell Reaction

Lennert (1961) compares the human stem cell hyperplasia or the large pyroninophilic cell reaction (24) to the proliferation of large lymphoid cells in the rabbit lymph node tertiary nodules (33) and to the pyroninophilic stem cell reaction in the guinea pig lymph node cortical pulp (18) Thus, these cells should also correspond to the large pyroninophilic cells and blasts (28 29) in paracortical, thymus-dependant areas (30) since all these cellular reactions in experimental animals are elicited by antigenic stimuli which are assumed to lead at least to a delayed type of hypersensitivity (DH) This response by some has been regarded to differ from the pure circulating antibody response elicited by a few antigens and consisting of rapid follicular activation and medullary plasmacytosis (28, 29) On the other hand, in several investigations using various bacterial and other antigens, a proliferation of large pyroninophilic cells preceded the development of active germinal centres and a mature plasmacytosis both in lymph nodes (2 7 32) and in the corresponding areas in the spleen (8, 16) Furthermore *Davies et al* (5, 6) found quantitative differences only in the early blast reaction of the mouse lymph node response both to sheep erythrocytes (circulating antibodies) and to oxazolone (DH) Thus, using the terminology of *Turk & Oort* (1967) an immunological lymph

TABLE 5 *The Degree of Large Pyroninophilic Cell Reaction (Large Pyroninophils) a... and the Y e Agglutinins, the Y e Antibodies Detected by Immunofluoresce...*

Case	2				3				4			
Day	1	6	26	47	1	5	18	52	1	6	20	48
Lymph node												
Large pyroninophils	+								+ +			
Germ centre activity	±								+ +			
Y e agglutinin titres	320	5120	320	160		<20	160	20	2560	1280	640	320
Y e antibodies by imm fluorescence												
IgM	20	1280	80	20		–	20	5	320	80	20	20
IgG	–	320	80	80		–	5	5	80	80	20	20
IgA		320	20			–			–	–		
IgD	–	–	–	–					–			
Serum immunoglobulin levels												
IgM	76	80		88		60	60	25	108	75		72
IgG	2250	2030		2050		1100	1550	1180	1560	1640		1400
IgA	298	260		208		265	350	240	28			<10

Piringer s lymphadenitis (*e g* toxoplasmosis) in infectious mononucleosis and in lymph node hyperplasias of unknown aetiology, especially in the head and neck and perhaps caused by rubeola Similar cells have been described as atypical cells or atypical mono nuclear histiocytes in infectious mononucle osis and after vaccination (31) These cells are easily recognized in well stained methyl green pyronine sections and immediately they were recognized we started to see them occasionally in varying but usually small numbers at the margins of carcinomas in a multitude of chronic inflammations and also in the interfollicular pulp of tonsils and adenoids

Concerning the differential diagnosis of large pyroninophilic cell reaction malignant lymphomas (17 31) and especially Hodg kin s disease (17) should be taken into ac count According to *Lennert* (1961) such a cellular proliferation may occur without notable germinal centre activity in the early stages of lymphadenitis our own observations fit this statement Thus the absence of ger minal centre activity should not indicate malignancy unless features other than the proliferation of large pyroninophilic cells arouse suspicions in this direction at least if the difference between malignant and benign growth is as distinct as is usually supposed

Clinicopathological Aspects in Enteric Infection with Y e

In our cases enteric infection with *Y e* was usually associated with a macroscopically normal but microscopically focally ulcerated appendix and sometimes with pronounced proliferation of large pyroninophilic cells without reticulocytic abscesses in the mesen teric lymph nodes The reactions are *not* specific for *Y e* but in patients presenting such alterations associated *with* abdominal symptoms this infection should be kept in mind It cannot be excluded *with certainty* that reticulocytic abscesses might have de veloped after the biopsies *had been* taken Germinal centre micro abscesses observed by others (38) in the appendix in this infection were seen in only 1 out of 7 cases Subserosal granulomas (23) were not found

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	80	80	80	80	5	80	20	80	5	80	320	20	5
–	5	320	320	320	320	20	20	80	20	320	320	320	80
	–	20	–	320	20	80	80	20		5	20	–	–
		–	–	–	–	–	–	–	–	–	–	–	–
56	64	76	38	150	180	171	108	124	156	139	110	150	
940	1270	1570	1250	1560	2080	1500	1630	1730	1740	930	1200	1250	
165	220	187	174	220	185	135	146	124	140	130	160	155	

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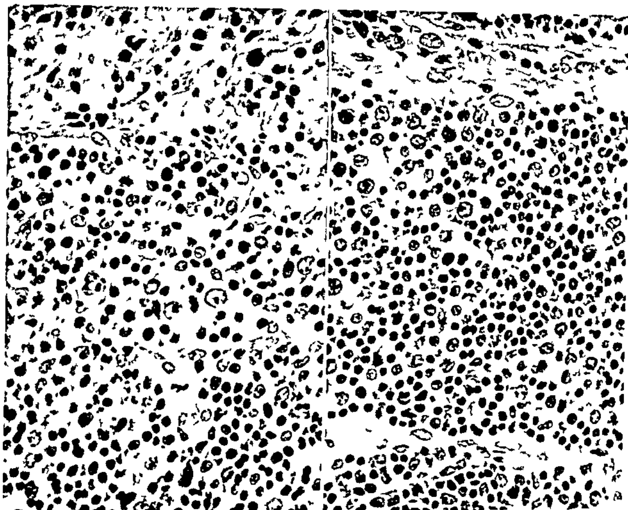


Fig 5 Large and mitotically dividing cells in the marginal sinus in case 7 (left) and in an efferent lymphatic vessel in case 6 (right) stained with H & E × 515

node response in experimental animals often seems to start as a DH type of response turning rather rapidly into a mixed one ending as a pure circulating antibody response This sequence closely resembles that in human non specific lymphadenitis as described by *Lennert* (1961) It is not known whether the patients simultaneously developed DH against *Y e* At least in those cases in which *Y e* could be cultivated from the node the antigen must have been present in a particulate form, in all these cases there was a proliferation of large pyroninophilic cells

Davies et al (6) found follicular activity to coincide as to time with the appearance of antibodies in the serum This should not be considered to indicate that follicular activity or germinal centres are needed for the production of at least some circulating antibodies since the latter appear in the serum before a noteworthy activation of spleen germinal centres (16) even if this has been prevented by irradiation (13) Furthermore antibodies reach the peak in the regional lymph nodes proper earlier than in the serum (7) and phylogenetically the IgM response does not seem to require the participation of germinal centres (10) Evidence supporting a different cellular origin of 19 S and 7 S antibodies has been reviewed (11 21) and also later studies (8 25) indicate that the former often are formed by cells other than mature plasma cells The agglutinins and haemolysins formed concomitantly with a proliferation of large pyroninophils (5 7 13 16) were probably IgM (*e g* 21)

During the primary immune response lym

phoid cells are released from the lymph nodes and the spleen (7, 8) to colonize other organs. Such cells might correspond to the large lymphoid cells seen in the sinuses and the efferent lymph vessels in some of our cases and perhaps to the cells regarded as plasma cells in the blood of the two patients on whom a differential count was performed

Originally familiar only with the works of *Oort & Turk* (1965) and *Parrot & de Sousa* (1966) we found it difficult to explain why the formation of specific circulating antibodies, in part consisting of IgM, was associated with a lymph node response which, according to these authors, morphologically seemed to have started as a DH type of response Most of the experimental studies cited, however, explain this association and also other features found in the lymph nodes of our cases Lymph node responses in experimental animals and in man (17) seem to follow the same sequence of events and perhaps one should try, in this respect, to bring the nomenclatures of immunology and pathology in conformity with each other?

Even in man, large pyroninophilic cells at the site of the cortical pulp postcapillary venules might develop from small lymphocytes (24) According to *Davies et al* (5 6) in the early immune response of the mouse, a blast and mitotic response of thymus derived cells precedes a similar reaction of bone marrow derived cells but it seems difficult to distinguish these types of cells from each other morphologically The origin of these cells in man does not seem to have been established (24)

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Renin in Intraocular Endometrial and Myometrial Transplants

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The question whether renin formation in the rabbit uterus takes place in the endometrium the myometrium or in both has been touched in a previous paper (*Eskildsen* 1970) The results indicated a synthesis of renin in both, but a transport of renin from one layer to the other during the experiment was not eliminated A solution of the problem is aimed at in the present paper, the purpose of which has been to show if there is a renin formation in transplants of isolated endometrial and myometrial tissue For this purpose small pieces of endometrium and myometrium were autotransplanted to the anterior chamber of the rabbit eye, using a method described by *Schochet* 1920 and later used for the study of rhytmic vascular changes in the endometrium of rabbits during pregnancy (*Markee* 1930, *Krichesky* 1943) and after stimulation with estrogens (*Kaiser* 1948) With this method it is possible to follow the fate of the grafts and macroscopically estimate their vitality before removal for renin estimation and histological studies

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MATERIAL AND METHODS

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propylene glycol in doses of 50 μg per kg body weight subcutaneously twice daily for 2 days, while the remaining 4 animals only received propylene glycol. Grafts and uterine biopsies were removed 12 hours after the last injection. Finally, 4 animals in *Group III* received transplants of autologous ureter tissue, 4 pieces into the one eye. Two of them further received autotransplants of 4 pieces of renal artery into the other eye. These grafts were all removed 30 days after the transplantation.

Transplantation technique—The animals were anaesthetized with pentobarbital sodium intravenously (40 mg/kg body weight) and killed by air intravenously in connection with removal of the grafts. Uterine tissue for transplantation was obtained by removal of a segment of one uterine horn through a laparotomy. After immediate opening antimesometrially, small flakes from the endometrial and myometrial surface were isolated by help of a little sharp dissection scissor. 4 flakes of each sort, each measuring about 1 × 2 × 4 mm were then placed in the anterior chamber of the eye through an about 3 mm long incision: endometrial grafts on the left side and myometrial on the right side. In several cases, uterine tissue removed at the transplantation was used for renin determination in order to compare the renin concentration in the uterus at the moment of transplantation with that of the uterine tissue removed simultaneously with the removal of the grafts (Fig. 2).

Behavior of grafts and animals after transplantation—One to two days after the transplantation the grafts were found to be haemorrhagic and black with variable signs of conjunctivitis and keratitis which were treated with chloramphenicole ointment. In the course of 8 to 14 days the reaction declined in all cases and the grafts showed marked signs of regeneration accordingly as implantation on the iris occurred. This was especially the case in the endometrial grafts, which were of an orange or pink colour and sometimes contained macroscopically visible vessels. The myometrial grafts were more whitish fibrotic looking but in all cases they were implanted to the iris.

In the first week after transplantation the animals were blind, but in spite of that there were no problems with the nutrition. After that time the animals showed signs of returning vision in most cases on both sides. Their general behaviour now seemed unchanged from normal.

Removal of grafts and uterine tissue—After removal from the eye the grafts were carefully dissected free from the underlying iris tissue and divided into two equal halves. The four different halves were pooled and used for renin determination and histological examination respectively. Simultaneously a segment of the remaining untouched horn was removed, immediately opened antimesometrially and frozen in a mixture of isopentane and dry ice (—65° C), whereafter flakes of frozen endometrium and myometrium were isolated as described previously (*Eskildsen* 1970). In 6 cases the remaining iris tissue which had not been in direct contact with the grafts was removed separately from the two eyes and used for renin estimation.

Weight of the grafts—In several (16) cases the grafts were weighed just before application into the eye and it was found that the weight of 4 grafts, each measuring about 1 × 2 × 4 mm was rather constantly about 20 mg. The weight of the same transplants measured just after removal from the eye, was diminished a little, to about 15 mg, when the animals were left untouched but to about 10 mg when ovariectomy had been performed. Furthermore it was observed that a weight loss due to freeze drying took place during storation at —20° C. The values for the renin concentration were therefore corrected to give the concentration found in the grafts just after removal from the eye. As regards some of the animals in Group I, the grafts were only weighed after shorter or longer storage at —20° C and consequently in this group the renin was indicated as the renin *content* in Goldblatt Units of the four grafts.

Tissue extraction, homogenization and transitory acidification performed to inhibit angiotensinase activity were performed as mentioned previously (*Eskildsen* 1970).

Renin assay combined with radio immuno assay was based on the principle of determination of the decrease in angiotensinogen concentration in course of time. 150 μl of tissue extract diluted by 0.2 M tris/HCl (pH 7.5) to a renin concentration about 1×10^{-4}–1×10^{-3} Goldblatt Unit (G.U.) per ml, was incubated for 18 hours at 37° C with 50 μl of a fixed concentration of renin substrate (ca. 2000 ng/ml). The angiotensin concentration before and after incubation was estimated by a stoichiometric transformation to angiotensin II which was measured by radio immuno assay (*Poulsen* 1969). The renin concentration was expressed in G.U./ml by reference to a hog renin standard preparation kindly supplied by dr. Haas and identical with that donated by dr. Haas to the WHO Laboratory for Biological Standards (Nat. Inst. Med. Res., Mill Hill (London)). A plasma pool from 24 hours nephrectomized rats before use transitorily acidified to inhibit angiotensinase activity was used as renin substrate. $Asp(NH_2)^1$-val^5 angiotensin II (Ciba pure substance) which on weight basis was found identical to the Mill Hill preparation was used as angiotensin standard.

Histological examination was performed on the normal uterine tissue as well as on the grafts from every animal. In all cases one half of the grafts was examined, but in 6 cases both halves of the grafts were used for histological study. The tissue

was fixed in Helly's solution for 24 hours, paraffin embedded cut in 3–4 micron thick sections and stained by haematoxylin-eosin van Gieson and PAS (cold procedure, *Petri* 1969)

RESULTS

1 *Renin Concentration in Normal Endometrium and Myometrium from Mature Rabbits*

The renin concentration in the isolated endometrium and myometrium from 14 mature animals used for transplantation

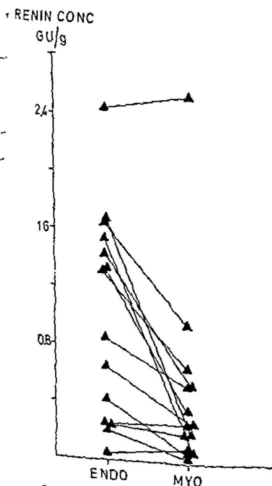


Fig 1 The distribution of renin (G U /g) in situ studied on isolated endometrium (ENDO) and myometrium (MYO) of 14 animals The lines connect the corresponding individual values

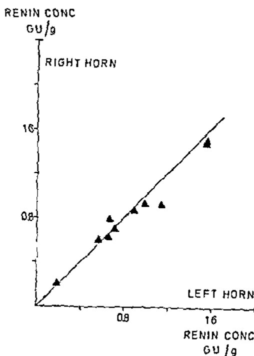


Fig 2 The accordance between renin concentrations in G U /g, in biopsies from the two uterine horns, the left removed simultaneously with the transplantation procedure, the right simultaneously with removal of the intraocular grafts

showed marked individual variations The concentration in the endometrium ranged from 0 05–2 45 G U /g and in the myometrium from 0 03–2 50 G U /g (Fig 1) With a few exceptions the renin concentration was higher in the endometrium than in the myometrium

During control of the variability of the renin concentration 1) in the two uterine horns and 2) in the uterus during the time used for the transplantation experiment, estimations were performed on uterine tissue removed after an interval of 7–28 days from the two horns in connection with application as well as removal of the grafts Correlation between these two values in 10 normal animals showed, Fig 2, that the renin concentration was almost identical in the two horns and unchanged during the experiment

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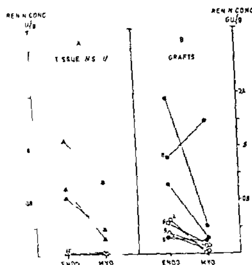


Fig 4A The effect of treatment with oestradiol monobenzoate 50 µg/kg (▲) or the dilution medium propylene glycol (Δ), injected subcutaneously twice in 2 days on the renin concentration (G U /g) of endometrium (ENDO) and myometrium (MYO) *in situ* from 6 castrated animals

Fig 4B The effect of the same treatment on endometrial (ENDO) and myometrial (MYO) grafts (estradiol marked ● — propylene glycol marked ○) in 8 animals, castrated 14 days after the transplantation The tissue *in situ* belonged to six of the transplanted animals (indicated by the animal no) and was removed simultaneously with the grafts

from 0 01–0 13 G U /g in myometrial grafts Concentrations were higher than those in the endometrium and myometrium *in situ* *Estradiol treatment* (performed as subcutaneous injection of oestradiol monobenzoate 50 µg/kg twice daily for two days) of 4 castrated animals (Group II) resulted in a significant increase in the renin concentration in the tissue *in situ* as well as in the grafts (Fig 4A + B) The effect of estradiol treatment was most remarkable in the uterine tissue *in situ* the renin concentration both in the endometrium and myometrium being at the level otherwise seen in normal mature animals (Fig 1) As to the grafts three pairs showed an increase in renin content distinctly above the castrate values even though they were below the values in the grafts of normal mature animals (Fig 3A) The fourth pair of grafts did not respond to the estradiol stimulation, the content in the endometrium being only 0 24 G U /g and in the myometrium only 0 19 G U /g (Table 1)

4 *Intra Ocular Autotransplants of Ureter and Renal Artery*

The purpose of this experiment (Group III) was to study whether the transplantation procedure and the implantation of grafted tissue into the eye might be able to stimulate a formation of renin in tissue which under normal conditions did not seem to produce renin The renin concentration in the normal ureter and renal artery was very low, indicating that no renin synthesis took place in the tissue *in situ* (Table 2) The ureter grafts, which macroscopically behaved just as the endometrial grafts during the four weeks after transplantation, showed no signs of renin formation except in one case in which the renin concentration for some unknown cause was significantly elevated above the normal level, even though it was markedly below the renin concentration found in uterine grafts The grafts of renal artery remained whitish, fibrotic after transplantation to the eye and resulted in a contraction of the iris The renin concentration of these grafts was unmeasurable

5 *Occurrence of Renin in Iris Tissue from Grafted Eyes*

In 6 animals from Group I the remaining iris tissue, after removal of the transplants, was analysed for renin In almost all cases, the renin concentration in iris tissue from the left eye, used for endometrial transplantation, was markedly elevated above the concentration found in normal iris tissue, 0 002 G U /g (Table 3) In contrast, iris tissue from eyes grafted with myometrial tissue showed in all cases but one no significant increase in renin concentration

2 *Renin Content in Intra Ocular Transplants of Endometrium and Myometrium in Normal Mature Rabbits*

In the case of 16 animals (Group I) grafts transplanted at intervals varying from 2 to 57 days showed a great variation in the content of renin (Fig 3A) It was remarkable that, in course of the first week of transplantation, the renin content in endometrial and myometrial grafts was rather low, being 1 2 × 10⁻³ G U and 0 4 × 10⁻³ G U respectively after 2 days and 4 2 × 10⁻³ G U and 0 4 × 10⁻³ G U after 4 days Only 7 days after transplantation, when the grafts showed signs of regeneration, the renin content had increased markedly and the values were not distinguishable from the values of grafts transplanted for longer time The renin content in the grafts showed great individual variations, like the renin concentration in the normal tissue (Fig 1) In 7 animals in which the transplants were removed after 14 days, the renin content in endometrial grafts ranged from 9 × 10⁻³–123 × 10⁻³ G U and in myometrial grafts from 1 × 10⁻³ — 45 × 10⁻³ G U Thus, in conformity with findings in normal tissue (Fig 1) the grafts showed in almost all cases a higher renin content in the endometrium than in the corresponding myometrium

Fourteen days after transplantation, the renin *concentration* in grafts in 3 of the animals was compared with that of the normal tissue (Fig 3B) It was in this way shown that the renin concentration was markedly higher in the grafts than in the tissue *in situ* the ratio of the renin concentration in these two places ranging from 3–4 in the endometrium and from 1 5–7 in the myometrium (Table 1) A comparison between these values and those of grafts removed only 2 and 4 days after transplantation (Fig 3B) showed how the renin formation declined in the grafts just after application into the eye but increased again when implantation and regeneration had occurred

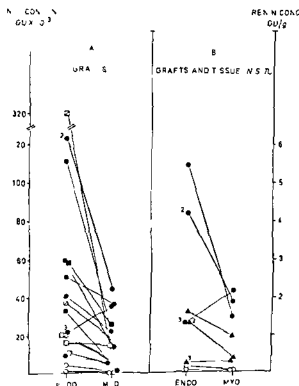


Fig 3A The renin *content*, G U ×10⁻³ per 4 grafts of endometrial (ENDO) and myometrial (MYO) tissue removed 2–4 (○), 7 (⊘) 14 (●) 28 (□) 46 (⊠) and 57 (■) days after transplantation *Fig 3B* The renin *concentration* (G U./g of endometrial (ENDO) and myometrial (MYO) grafts of the animals transplanted in 2-4 days (○) and 14 days (●) As comparison is shown the renin concentration in endometrium and myometrium *in situ* (▲) from the animals (indicated by the no 1–3) transplanted in 14 days The lines connect the corresponding values in the grafts as well as in the tissue *in situ*, of every animal

3 *Effect of Ovariectomy and Estradiol Treatment on Grafts of Endometrium and Myometrium*

The renin concentration in the endometrium and myometrium *in situ* decreased remarkably after *ovariectomy* (Fig 4A) This was observed in three of the four control animals in Group II which four days after ovariectomy were injected twice daily for two days with propylene glycol This effect of ovariectomy was also clearly shown on the grafts (Fig 4B), the renin concentration of which, compared with that in grafts from normal mature animals (Table 1) was markedly decreased The values ranged from 0 26–0 51 G U /g in endometrial grafts and


gr
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TABLE 1 *Comparison between Renin Concentration in Tissue* in situ *and Transplanted Uterine Tissue from Normal Mature Animals, Castrated and Castrated Estradiol Treated Animals*

Animals	No	Tissue *in situ* G U/g		Grafts G U/g	
		Endometrium	Myometrium	Endometrium	Myometrium
Normal	1	1 65	0 96	5 48	1 47
Mature	2	1 34	0 37	4 22	1 88
	3	0 26	0 27	1 34	2 17
Castrated	4	0 092	0 016	0 51	0 01
Control	5	0 019	0 019	0 31	0 11
	6	0 015	0 010	0 46	0 13
	7	–	–	0 26	0 04
Castrated	8	1 72	1 09	1 44	1 99
Estradiol	9	0 86	0 38	0 24*	0 19*
Treated	10	1 00	0 23	1 04	0 24
	11	–	–	2 33	0 42

Record of individual values of the renin concentration (Goldblatt Unit per gram tissue) in endometrium and myometrium from 11 animals to which grafts in all cases had been transplanted 14–20 days earlier The castrated animals estradiol treated and controls, were injected simultaneously twice daily for two days with oestradiol *monobenzoate 50 µg/kg and the dilution medium propylene glycol* respectively The values marked * are within the castrate level

6 *Histological Examinations of Uterine Grafts*

The microscopical study of the one half of every *removed transplant established that the* grafts consisted of absolutely pure fractions of endometrium or myometrium In six cases where both halves were examined it was further found that the one half was representative for the whole graft

The macroscopically observed haemorrhage in the grafts shortly after application to the eye *resulted in corresponding changes in the* microscopical picture Two days after transplantation, the endometrial grafts showed marked haemorrhagic necrosis with loss of

Fig 5A Endometrium *in situ from a normal* mature rabbit The uterine glands (gl) are enclosed in the luminal part of the endometrium The stroma beneath the glands is composed of connective tissue containing mesenchymal stroma cells and vessels, especially small venoles (v) and arterioles (a) in the deeper part A few mononuclear *cells* are ordinarily observed in the endometrial stroma Haematoxylin eosin stain 300 ×

Fig 5B Myometrium *in situ* from the same animal *The muscle bundles are arranged* in two layers a peripheral longitudinal one (ml) and a central circular layer Connective *tissue containing* large arterioles (A) and venoles (V) is placed between the two layers Haematoxylin eosin stain 300 ×

Fig 6 Endometrial (6A) and myometrial (6B) intra ocular grafts removed 2 days after application *to the eyes* The *endometrial* tissue is almost without structures and flooded with erythrocytes (e) No stroma cells are seen and *the vessel walls* (w) are destroyed without visible endothelial or smooth muscle cells Haematoxylin eosin stain *300* ×

The bundles of muscle cells in the myometrial (6B) graft are thin wavy in some places without visible nuclei and surrounded by a fibrinoid structureless substance Van Gieson 300 ×

Fig 7 Endometrial (7A) and myometrial (7B) grafts 7 days after the transplantation The glandular structures and the stroma cells in the endometrial graft show signs of regeneration Haematoxylin eosin stain 300 × In the myometrial graft *the smooth muscle cells* (grayish *nuclei*) have regenerated but the content of connective tissue and *fibroblasts (black nuclei) is increased* in comparison with the myometrium *in situ* Van Gieson stain *300* ×

TABLE 4 *Semiquantitative Evaluation of the Vitality of Endometrial Grafts Compared with the Renin Content*

Duration of transplantation	Signs of regeneration: Number and size of stroma cells	Signs of regeneration: Vascularization	Signs of degeneration: Haemorrhage	Signs of degeneration: Fibrosis	Renin content G U × 10⁻³ per 4 grafts
14 days	+ + + +	+ + + +	—	—	111
14	+ + +	+ +	—	—	33
14	+	+	+ + +	+ +	9
28	+ + +	+ + +	—	+	20
28	+ +	+ +	—	+ +	16
46	+ + +	+ + +	—	—	320
46	+	+	+ + +	+ + +	4

The symbols + → + + + + indicate increasing signs of regeneration or degeneration while — indicates lack of such signs. The histological estimation is in every case based on the evaluation of 4 grafts performed independently by two persons who did not know the renin content of the grafts concerned.

out any PAS positive secretion a decreased vascularization and decreased number of stroma cells which seemed to be smaller and more dark than normal. In contrast to this estradiol treatment (Fig 9B) resulted in a high prismatic gland epithelium containing PAS positive secretion and a great content of vessels and large light stroma cells.

DISCUSSION

Recent investigations of the distribution of renin in the endometrium and myometrium of the rabbit uterus showed a marked content of renin in both places in the normal mature rabbit (*Bing & Eskildsen* 1968) as well as in the pregnant animal approaching the end of pregnancy (*Bing & Faarup* 1966) and in the castrated animal after few hours stimulation by estradiol (*Eskildsen* 1970). The renin concentration was most often higher in the endometrium than in the myometrium and a transport of renin from one layer to the other was not eliminated even if it seemed rather unlikely.

In the present study the renin content in flakes of isolated endometrium or myometrium was studied after transplantation to the anterior chamber of the rabbit eye.

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After a preceding destruction of the tissue with cessation of renin formation during the first week after transplantation the grafts of endometrium as well as myometrium were implanted on the iris and showed a marked but individually varying formation of renin which even exceeded the formation in the corresponding tissue *in situ* from the same animals.

It was further found that castration resulted in a marked decrease in the renin formation of the grafts of both endo and myometrium and that the values increased after subsequent estradiol treatment although the renin concentration did not reach quite the same level as that found in grafts in normal animals. Furthermore in normal animals as well as in castrated ones it was characteristic that the renin concentration was higher in the grafts than in the tissue *in situ* from the same animals indicating that the renin formation in the grafts was exposed to a stimulation the cause of which is unknown.

The finding of renin in extracts of the iris of eyes containing endometrial grafts but not (or only seldomly) in eyes with myometrial grafts indicate a diffusion of renin from endometrial grafts but not from the myometrial grafts possibly because of the

TABLE 2 *Comparison between the Renin Concentration in Tissue in situ and Grafts of Ureter and Renal Artery*

	Animal no	Tissue in situ G U/g	Grafts G U/g
Ureter	1	0 002	0 082*
	2	0 003	0 002
	3	0 004	0 003
	4	0 005	0 004
Renal Artery	2	0 006	0 003
	3	0 003	0 003

In all the animals the grafts were removed 30 days after transplantation The value marked * was the only one which was significantly elevated above the values in tissue *in situ*

structures, glands and vessel walls and flooding of the main part of the grafts with erythrocytes (Fig 6A) In the myometrial grafts the degeneration was remarkable *in the loss* of nuclei in the muscle cells simultaneously with deposition of a fibrinoid substance around the graft and between the muscle bundles (Fig 6B) These changes were still marked after four days, but *signs of a beginning* regeneration were observed, after seven days almost all the necrotical changes had disappeared (Fig 7A + B), from that

TABLE 3 *Content of Renin in Iris Tissue after Implantation with Grafts of Endometrium and Myometrium*

	Animal no	Left eye Endometrial site G U/g	Right eye Myometrial site G U/g
Implanted Iris	1	0 005	0 018
	2	0 011	0 006
	3	0 038	0 007
	4	0 016	0 007
	5	0 051	0 011
	6	0 051	0 003
Normal Iris	7, 8, 9	0 002	

The values in the table indicate the renin concentration (G U/g) The grafts with underlying iris tissue were removed before estimation was performed on the remaining part of the iris

time and until the 57th day after transplantation most grafts showed normal structure and only a few grafts still included haemorrhage or widespread fibrosis When organization had taken place, the stroma of the *endometrial* grafts in contrast to the tissue *in situ* (Fig 5), contained a great number of mononuclear cells plasma cells and some granulocytes besides the ordinarily occurring stroma cells Furthermore it was remarkable to notice the large meshes and the thinness of the framework of reticular fibres in the transplanted stroma as compared with features in the normal tissue The distribution of vessels was almost as that in a normal endometrium but the number of vessels was in most cases more scarce The *myometrial* grafts were characterized by fibrosis which encapsulated the muscle tissue

Examination of the arterioles in endometrial and myometrial grafts stained with PAS showed lack of granulated epithelioid cells (like those found in the juxtaglomerular apparatus of the kidney)

In an attempt to explain the great variations in renin contents in grafts a semiquantitative evaluation of the morphological signs of organization, regeneration and degeneration was performed (and controlled by a colleague) on grafts with different contents of renin If grafts were compared at identical intervals after transplantation it was possible in endometrial grafts (Table 4) to find some correlation between high renin content and a great number of large light stroma cells combined with a well established vascularization (Fig 8B) Haemorrhage and fibrosis of the tissue were found in grafts with a lower renin content (Fig 8A) A similar evaluation was attempted in the study of the myometrial grafts but without finding any correlations

The effect of *castration* and following *estradiol* treatment was marked on the endometrial grafts while it was impossible to find significant changes in the morphology of the myometrial grafts After castration the endometrium (Fig 9A) was characterized by a low prismatic epithelium of the glands with

TABLE 4 *Semiquantitative Evaluation of the Vitality of* Endometrial *Grafts Compared with the Renin Content*

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out any PAS positive secretion a decreased vascularization and decreased number of stroma cells which seemed to be smaller and more dark than normal In contrast to this estradiol treatment (Fig 9*B*) *resulted* in a high prismatic gland epithelium containing PAS positive secretion *and a great content* of vessels and large light stroma cells

DISCUSSION

Recent investigations of the distribution of renin in the endometrium and myometrium of the rabbit uterus showed a marked content of renin in both places in the normal mature rabbit (*Bing* & *Eskildsen* 1968) as well as in the pregnant animal approaching the end of pregnancy (*Bing* & *Faarup* 1966) and in the castrated animal after few hours stimulation by estradiol (*Eskildsen* 1970) The renin concentration was most often higher in the endometrium than in the myometrium and a transport of renin from one layer to the other was not eliminated even if it seemed rather unlikely

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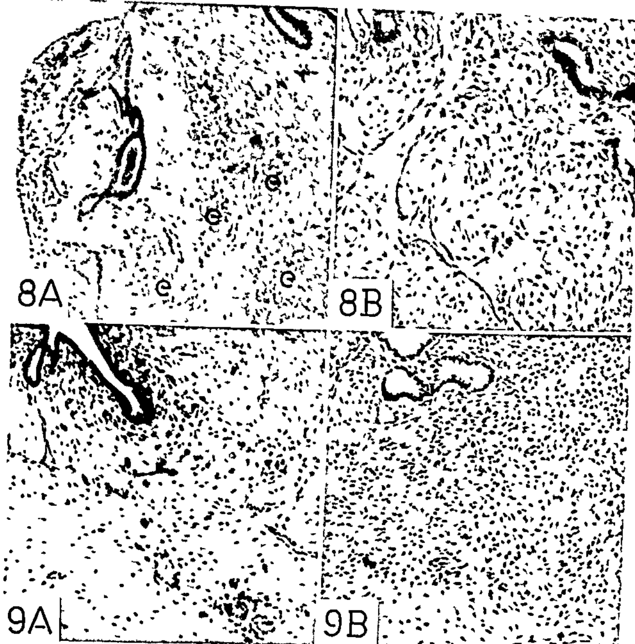


Fig 8 Endometrial grafts 14 days after the transplantation, in 8A with a low renin content (9·4 G U $\times 10^{-3}$–Table 4) and in 8B with a high renin content (111 G U $\times 10^{-3}$–Table 4) The tissue in 8A is characterized by haemorrhage (e–erythrocytes) and a stroma containing few vessels and stroma cells Haematoxylin eosin stain 300 × In opposition to this the tissue in 8B contains plenty of stroma cells with large, light nuclei and the vascularization is well developed PAS stain 300 ×

Fig 9 Endometrial grafts from animals castrated 14 days after the transplantation and four days later treated with propylene glycol (one case (9A)) another case (9B) being treated with estradiol diluted in propylene glycol In contrast to 9A the tissue in 9B is characterized by a high prismatic glandular epithelium, large closely packed stroma cells and a well developed vascularization Haematoxylin eosin stain 300 ×

fibrotic capsule which surrounded these grafts

Similar transplantation of small pieces of ureter or renal artery which both contained almost unmeasurable concentrations of renin did only in a single graft of ureter tissue show an elevated content of renin after the transplantation In all the remaining grafts of ureter or renal artery no increase in the renin content was observed

On the basis of these results it can be concluded that renin formation takes place both in the endometrium and in the myometrium In connection with this finding it was tried to see whether the histological findings in grafts with highly differing renin content could give an impression of the location of renin formation in the grafts In the endometrial grafts it was possible to find a sort of correlation between the renin content and the vascularization and number and size of stroma cells These findings may only be indications of the vitality of the grafts and do not allow any further conclusions about the cellular localization of the renin synthesis In the myometrial grafts there was no similar correlation between the renin content and the vascularization or any of the other structures

Cells like the renin producing epithelioid cells in the juxtaglomerular apparatus of the kidney were not found in connection with the vessels neither in the endometrium nor myometrium

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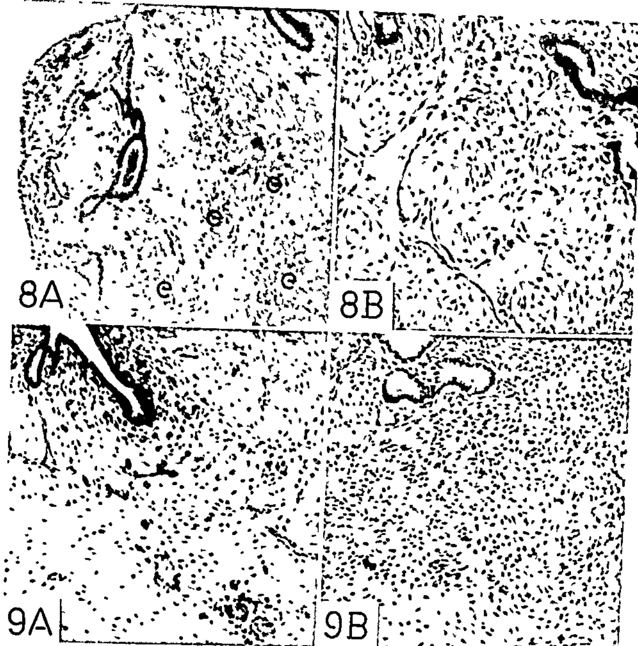


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cotized animals Blood was taken 21 to 24 hours after the operation by decapitation the blood being in most cases pooled from 3 mice, but in some from 2 or 5 animals In a few cases so much blood was obtained from single mice, that it was not necessary to use a pool 50 µl 6 per cent sodium citrate were added per ml blood which was taken in cooled tubes

Plasma renin concentration was measured as described by *Poulsen* (1969) The principle consists in a determination by radio-immuno-assay of the velocity of the decrease in the angiotensinogen concentration in the course of time The method is characterized by a precisely determined concentration of angiotensinogen during the reaction, controlled activity of the converting enzyme and complete abolishment of the influence of the angiotensinases The renin concentration (k E) is expressed in hours[1] and calculated from the equation

$$\ln \frac{S_o}{S_o - y} \quad (k\ E)\ t$$

S_o is the initial angiotensinogen concentration and $(S\ y)$ the angiotensinogen concentration at the time t The *angiotensinogen concentration*, expressed as nanograms angiotensin II per ml, was measured by a quantitative transformation to angiotensin II with excess of renin and converting enzyme and abolished angiotensinase activity The angiotensin II was measured by radio-immuno-assay and expressed as nanograms of angiotensin research Standard A from Mill Hill London For the preparation of the renin + converting enzyme preparation mouse serum or plasma enriched with mouse submaxillary renin were used The mouse renin was prepared by extracting previously 3 times frozen and thawn submaxillary glands from male albino mice with a pyrophosphate buffer 0 02 M pH 5 3) The angiotensinases of an extract 1 100 containing 14 Haas Goldblatt units per ml were eliminated by subjection to pH 3 6 for 20 minutes at 25° C Mouse angiotensin II reacts well with the antibody used for the radio-immuno-assay However the apparent adsorption of angiotensin II to proteins is more pronounced in mouse than in rat plasma This influenced the separation of free and bound angiotensin II and rendered it necessary to use a two times increase of the charcoal concentration and a five times decrease of the dextran concentration In some cases the transformation was performed under similar conditions but without inclusion of converting enzyme and with use of bio-assay on ansolysen pretreated rats instead of radio-immuno-assay The two methods were found to give identical results

Renal and *submaxillary renin* were determined after repeated freezing and thawing and extraction with pyrophosphate buffer by bio-assay in ergotamine tartrate pretreated, pentymal sodium narcotized rats, using the Haas standard from Mill Hill, London for comparison

The *DOCA and salt* treated mice received 6 mg DOCA in a microcrystal suspension (Percorten, Ciba) subcutaneously once a week and 1 per cent sodium chloride as drinking fluid for 5 to 6 weeks

RESULTS

1 *Submaxillary and Renal Renin*

The submaxillary and renal renin content (in units per 2 glands or 2 kidneys) was only determined in a limited amount of organs from the animals in which plasma renin and angiotensinogen were determined, as the aim of these analyses was to illustrate the differences in submaxillary renin content between the strains used in this study Fig 1 gives the

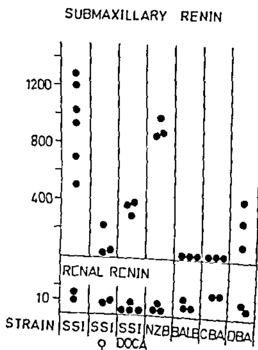


Fig 1 Submaxillary and renal renin (in Haas Goldblatt units per 2 glands or 2 kidneys) in male, and in one group of female, mice of different strains, including a group of DOCA and salt treated mice Note the difference between the two ordinate scales

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THE RENIN SYSTEM IN MICE

Effects of Removal of Kidneys or (and) Submaxillary Glands in Different Strains

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In different strains of mice the sum of renal and submaxillary renin was found to vary from about 10 to about 1000 Haas Goldblatt units about 7 to well over 98 per cent of which was found in the submaxillary glands. The plasma renin and angiotensinogen concentrations were independent of these variations Following *submaxillary sialo-adenectomy* there was only a slight decrease if any, in plasma renin or increase in plasma angiotensinogen Contrary to this, *nephrectomy* was followed by a marked decrease in plasma renin and a marked increase in plasma angiotensinogen, both of which were independent of the highly varying amount of submaxillary renin left in the organism The postnephrectomy increase in angiotensinogen was further independent of variations in the pre operative plasma and renal renin, supporting the previous conclusion that it must be due to loss of some internal factor other than renin or to loss of some external renal function

The renin system of mice differs in several ways from that of other animals It has thus been shown that the mouse submaxillary glands contain renin, the concentration of which is very high in some and much lower in other strains (*Werle et al* 1957 For further literature see *Page & McCubbin* 1968) It has further been shown that mouse angiotensinogen (renin substrate) is only split by mouse renin (*Oliver & Gross* 1966), and that mouse plasma converting enzyme and angiotensin I differ from those of the rat and the human renin system (*Poulsen & Bing* 1970)

The present paper which is part of a study of the cause(s) of the increase in plasma angiotensinogen (renin substrate) after nephrectomy aims at elucidating the effect of removal of kidneys or (and) submaxillary glands on plasma renin and angiotensinogen in mice of different strains including a group of desoxycorticosterone acetate (DOCA) and salt treated mice In this way it was possible to compare the changes in plasma renin and angiotensinogen produced by nephrectomy with those produced by removal of the submaxillary glands which in some strains contained about 100 times more renin and in other strains less renin than the kidneys

Received 21 viii 70

MATERIAL AND METHODS

Material 284 mice of the following strains 25 females from the strain C of the State Serum institute (S S I) Copenhagen weighing 28–40 g 119 males of the same strain weighing 25 50 g All the following mice were males 56 Balb and 18 NZB from the Medical Research Council Carshalton weighing 25–42 g 5 hybrides CBA NZB weighing 50–52 g 24 CBA from Chester Beatty Research Institute London weighing 38 45 g and 37 DBA/2J from Roscoe B Jackson Mem Lab, Bar Harbor U S A weighing 12 to 25 g

Operations after pretreatment with penicillin nephrectomy or (and) ablation of the submaxillary glands were performed in pentymal sodium nar

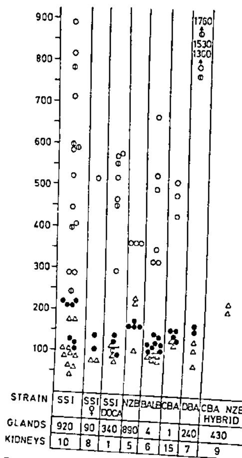


Fig 3 Plasma angiotensinogen (in ng angiotensin per ml) in male and in one group of female mice of different strains including a group of DOCA and salt treated mice Three values are seen to be higher than the scale being 1360 1530 and 1760 respectively The symbols and the information about the strains and the submaxillary and renal renin content are the same as in Fig 2

21 to 24 hours after *removal of the submaxillary glands* the angiotensinogen concentration was in most cases only slightly increased while 21 to 24 hours' *nephrectomized* mice had 3 to 15 times increased values The rather limited amount of pools from the different strains makes it difficult to say whether the differences between the strains are significant but it will be seen that the increases in angiotensinogen by nephrectomy induced are relatively small in NZB mice

and that the highest values are obtained in male S S I and especially in DBA mice The figure further shows that the female mice (only one pool containing blood from 5 mice) and the *DOCA + salt treated* male S S I mice show the same increase as untreated male S S I animals After *removal of both submaxillary glands and kidneys* the angiotensinogen concentration did not differ significantly from that found in nephrectomized mice

DISCUSSION

1 *In What Degree Does Plasma Renin Depend 1) on Submaxillary and 2) on Renal Renin?*

It is well known that the kidneys are the main site of renin formation in human beings and most mammals, and that plasma renin falls to very low values 24 hours after nephrectomy Contrary to this some mice have more than 98 per cent of their renin in the submaxillary glands and it might therefore be believed that removal of the submaxillary glands of such mice would result in the same decrease of plasma renin to the very low values found after nephrectomy on other animals This belief is supported by studies by *Takeda et al* (1969) who found that there was 1) a release of renin from the glands into the submaxillary veins 2) a higher plasma renin in male than in female mice which is in accordance with the sex differences in submaxillary renin (*Trautschold et al* (1966), *Oliver & Gross* (1967)), and 3) a significant reduction of plasma renin concentration (and of arterial blood pressure and size of the heart) 40 days following extirpation of the submaxillary glands in male mice

In the present study there was, however, neither any relation between submaxillary and plasma renin (Fig 2), nor any effect on plasma renin 21 to 24 hours after submaxillary sialo-adenectomy of mice belonging to the strain with high submaxillary renin (S S I) while—paradoxically—the plasma

results, showing that the *submaxillary* renin is very high in male mice of the S S I and NZB strain, about 10 to 25 per cent of these values in female S S I and male DBA mice and about 1 to 5 per mille of these values in male mice of the CBA and Balb strains In three DOCA and salt treated S S I mice the submaxillary renin is lowered to less than 50 per cent of that found in untreated mice

The *renal* renin (Fig 1) is seen to be much lower than the submaxillary renin in mice of the S S I and NZB strains In Balb mice, renal renin was about twice as high as and in CBA mice about 15 times higher than, the submaxillary renin DOCA and salt treated mice showed the usual decline to a very low renin content

From these findings it will be seen, that the *sum of submaxillary and renal renin* varies from about 1000 units in male S S I and NZB mice to about 10 to 15 units in Balb and CBA mice

2 *Plasma Renin*

The plasma renin content in pools of blood from about 3 mice was studied in blood from three strains of mice using both normal nephrectomized or (and) submaxillary sialo adenectomized mice as seen in Fig 2 The figure shows that the renin content in *normal mice* was 50 to 100 per cent higher in the DBA mice than in the two other strains *DOCA + salt treatment* of male S S I mice lowered the values to about one third of those found in untreated mice

21 to 24 hours after *removal of the sub maxillary glands* the renin content was un changed in both normal and DOCA + salt treated mice of the S S I strain and only little lowered in the two other strains

Nephrectomy resulted after 21 to 24 hours in a pronounced decrease of plasma renin in all three strains About the same decrease as in the nephrectomized was found in the S S I and the DBA strain after *removal of both kidneys and submaxillary glands* while these operations did not produce any meas urable reduction of the already low plasma

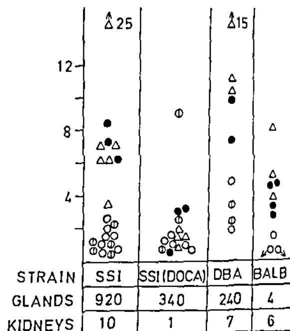


Fig 2 Plasma renin concentration (in hours^{-1} × 10^{7}) in male mice of different strains including a group of DOCA and salt treated mice The figure shows the values in pools of blood most often from 3 normal (Δ) submaxillary sialo adenectomized (●) nephrectomized (O) and sialo adenectomized as well as nephrectomized (⦶) mice Two values are seen to be higher than the scale being 25 and 15 respectively Below the information about the strains the figure shows the mean content of renin in H G Units per 2 submaxillary glands and 2 kidneys

renin levels in DOCA and salt treated ani mals

3 *Plasma Angiotensinogen*

Just as the renin determinations the *an giotensinogen* assays were performed on pools of blood from about 3 mice The values are given in Fig 3 which shows that while most strains had values between about 75 to about 125 two of them the NZB and the NZB CBA hybrid had higher values 200 to 225 The scatter of the values was most pro nounced in the males of the S S I Strain the highest values being found in the oldest mice (weight 45–50 g) It is further seen that the values of the female S S I strain and of the DOCA + salt treated male mice did not differ from that of the untreated male S S I mice

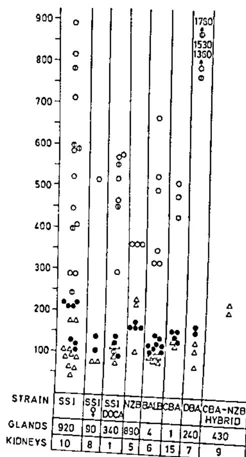


Fig 3 Plasma angiotensinogen (in ng angiotensin per ml) in male and in one group of female mice of different strains including a group of DOCA and salt treated mice Three values are seen to be higher than the scale being 1360 1530 and 1760 respectively The symbols and the information about the strains and the submaxillary and renal renin content are the same as in Fig 2

21 to 24 hours after *removal of the submaxillary glands* the angiotensinogen concentration was in most cases only slightly increased while 21 to 24 hours' *nephrectomized* mice had 3 to 15 times increased values The rather limited amount of pools from the different strains makes it difficult to say whether the differences between the strains are significant but it will be seen that the increases in angiotensinogen by nephrectomy induced are relatively small in NZB mice

and that the highest values are obtained in male S S I and especially in DBA mice The figure further shows that the female mice (only one pool, containing blood from 5 mice) and the *DOCA + salt treated* male S S I mice show the same increase as untreated male S S I animals After *removal of both submaxillary glands and kidneys* the angiotensinogen concentration did not differ significantly from that found in nephrectomized mice

DISCUSSION

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In the present study there was however, neither any relation between submaxillary and plasma renin (Fig 2), nor any effect on plasma renin 21 to 24 hours after submaxillary sialo adenectomy of mice belonging to the strain with high submaxillary renin (S S I) while—paradoxically—the plasma

renin of the more renin poor strain (DBA) and of the very *renin poor strain* (Balb) was slightly decreased Contrary to these differences between the results obtained by *Takeda et al* and those of *the present study* which, in any case, can be caused only in part by the *different times* of observation after sialo adenectomy, both investigations agree in the finding of *decreased plasma* renin after treatment with DOCA and salt

While the *submaxillary glands* thus played a *minor* role, if any, for the plasma renin in these mice the kidneys were found to play their usual role, known from other animals the plasma renin values being quite low 21–24 hours after nephrectomy The very doubtfull importance of the submaxillary glands for the level of the plasma renin is further demonstrated by the fact, that the values found after nephrectomy and those found after both nephrectomy and sialo adenectomy were about the same

2 *Effect of Submaxillary Sialo-Adenectomy or (and) of Nephrectomy on Plasma Angiotensinogen*

In a previous study on rats (*Bing* & *Poulsen* 1971) it was found that *the increased plasma angiotensinogen after nephrectomy is not secondary to changes in plasma and renal renin but must be due to loss of some internal factor other than renin or to loss of some external renal function* This conclusion is confirmed by the present study on mice (Fig 2) as shown by the following *three observations*

1) *removal of the submaxillary glands* containing from 50 to well over 98 per cent of the total submaxillary plus renal renin does only result in a slight increase if any in plasma angiotensinogen the increase being most evident in the strains with relatively little renin in the glands

2) *removal of the kidneys* results in the same marked increase in plasma angiotensinogen in mice as that found in other mammals, no matter whether the kidneys contain about 50 per cent or only less than 2 per cent of the total *renin* *It is further seen that there is no difference between the increase following nephrectomy and that following both nephrectomy and sialo adenectomy*

3) the postnephrectomy increase in plasma angiotensinogen *is about the same in untreated and in DOCA + salt pretreated mice,* independent of the marked differences between their pre operative plasma and renal renin content

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PRESENCE OF DMBA-^{3}H IN THE MOUSE OVARY AND ITS RELATION TO OVARIAN TUMOUR INDUCTION

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Ovarian tumours in mice can be induced by 9 10-dimethyl 1 2 benzanthracene (DMBA) DMBA's primary action in the mouse ovary is the destruction of small oocytes Whether the parent DMBA or some break-down product is the effective carcinogen and whether prolonged retention of the carcinogenic principle in the target organ is a prerequisite for a neoplasma to develop are unknown These questions were studied in experiments with tritiated DMBA comparing the primary cytological effect of DMBA with the presence of radioactivity in tissue extracts Radioactive compounds extracted from tissues after treatment with *DMBA ^{3}H* were analysed by two-dimensional chromatography The results *suggest* that DMBA itself is the effective initiator in ovary carcinogenesis in mice rather *than some in vivo* conversion of the chemical The different rates at which *small oocytes are destroyed* after oral and intraperitoneal administration of the carcinogen can be directly correlated with different levels of carcinogen obtained in the tissues after the two routes of administration and to their different excretion *rates It is concluded* that the carcinogenic *effect of* DMBA *on mouse ovaries is related to the immediate destruction of small oocytes,* whereas the prolonged retention of DMBA after intraperitoneal injection is of no significance for the neoplastic development

Ovarian tumours can be induced in certain strains of mice by 9 10 dimethyl 1 2 benzanthracene (DMBA) regardless of the route of administration (*Howell et al* 1954 *Marchant* 1957 *Biancifiori et al* 1961 *Krarup* 1967 *Kuwahara* 1967 *Shisa* & *Nishizuka* 1968 *Krarup* 1970a)

The primary action *of DMBA* in the mouse ovary is *destruction* of small oocytes *This* reduction starts shortly after application *of* the carcinogen and total depletion occurs at an early age (*Krarup* 1970*b*) Simultaneously with the regression *in the* oocyte population pathological changes develop in the ovaries, which ultimately give rise to large *granulosa cell* tumours (*Krarup* 1967, 1970a)

The question arises whether the effect on mouse ovaries is due to unchanged DMBA or to some metabolic product Furthermore it is unknown whether the effective compound immediately after application causes an oocyte destruction which secondarily leads to tumour *formation*, or *whether prolonged retention in* the ovaries is a prerequisite for *the tumour* development These questions have been investigated by *determining* the immediate cytological effect *of* DMBA on the small oocytes in relation *to the* uptake and excretion of tritium by ovaries and other tissues of mice treated with tritiated DMBA An attempt was *done to* identify tritium labelled compounds extracted from the tissues

Received 21 viii 70

MATERIALS AND METHODS

Preparation of DMBA ³H and Chromatographic Procedure

Female mice of the Bagg strain aged 21 days were used

9 10 dimethyl 1 2 benzanthracene (DMBA Fluka AG, Chemische Fabrik Buchs Switzerland) was prepared as a 0,5 per cent solution and a 0 1 per cent solution in olive oil for oral and intraperitoneal application respectively

DMBA generally labelled with tritium (DMBA ³H, Radiochemical Centre Amersham England, specific activity ranging from 8 0 Ci/mM to 18 6 Ci/mM radiochemical purity by thinlayer chromatography on Silica Gel in *n* hexane > 95 per cent) was dissolved in 0 5 per cent and in 0 1 per cent solutions of DMBA (carrier) in olive oil for oral and intraperitoneal administration respectively

The radiochemical purity of administered DMBA ³H and its possible degradation due to storage was evaluated by thinlayer chromatography 5 mCi of DMBA ³H in an evacuated ampul was dissolved in 2 ml of ether, transfered into a brown centrifuge tube containing 8 mg carrier DMBA and evaporated to dryness The compound was dissolved in 8 ml olive oil and stored at 37° C in the dark The concentration was approximately 0 1 per cent DMBA with specific activity of 625 μCi/ml A small amount of the solution was diluted 1 10 with benzene 3 times at 3 day intervals and thinlayer chromatography of 5μl was performed immediately after the dilution The chromatography was carried out in the dark on glass plates coated with 0 25 mm activated Silica Gel G nach Stahl (E Merck A G Darmstadt, Germany) (*Boyland & Sims* 1965) The chromatographs were developed in two dimensions the first one in hexane and the second one in benzene containing 5 per cent (v/v) of ethanol After chromatography in the first dimension the plates were examined in ultraviolet light to determine the position of the DMBA spot by its violet fluorescence Then chromatography was performed in the second dimension and two series of spots were studied The first series starts from the application spot and the second series starts from the fluorescent spot The position of DMBA in this chromatographic system was determined according to its R_F value (*Boyland & Sims* 1965) The two series were divided into eight equally sized areas which were scraped off the thinlayer plates into polyethylene vials Scintillator (0 4 per cent PPO and 0 005 per cent POPOP in toluene) was added and the specimens were counted in the scintillation spectrometer

Experiment A 1 (Effect on small oocytes of DMBA given orally)

Thirty six mice were anaesthetized with ether and 0,05 ml of the 0 5 per cent solution of DMBA (0,25 mg DMBA) was fed through a thin polyethylene catheter after gastric intubation

Experiment A 2 (Effect on small oocytes of DMBA given intraperitoneally)

Twenty seven mice were injected intraperitoneally with 0 25 ml of the 0 1 per cent solution of DMBA (0 25 mg DMBA)

21 untreated mice served as controls

In experiments A a number of mice were killed and their ovaries removed 24 hours 7 days 14 days, 21 days 28 days and 2½ months after treatment The ovaries were processed and counts of small oocytes performed as previously described (*Krarup* 1970b)

Experiment B 1 (Radioactivity in tissues after administering DMBA ³H orally)

Forty mice were fed 150 μCi DMBA ³H and 0 25 mg DMBA dissolved in 0 05 ml olive oil

Experiment B 2 (Radioactivity in tissues after administering DMBA ³H intraperitoneally)

Forty four mice were injected intraperitoneally with 150 μCi DMBA ³H and 0 25 mg DMBA dissolved in 0 25 ml olive oil

In experiments B mice were killed at different time intervals after the treatment from 6 hours up to 3 months (experiment B 1) or 5 months (experiment B 2) 2 to 6 mice were used at each observation

The animals were killed with ether Ovaries adrenals, samples of liver paravesical fat, femoral muscle, and brain were removed for examination

Preparation of Tissues and Counting Procedure

The specimens were weighed immediately after removal Lipids and lipid soluble compounds were extracted for 2–3 days in chloroform methanol 2 1 after homogenization The chloroform methanol was then evaporated and benzene added After centrifugation the supernatant containing that fraction of tissue radioactivity which is soluble in organic solvents (*lipid soluble fraction*) was assayed for radioactivity in a Packard Tri Carb liquid scintillation spectrometer using NE 220 (Nuclear Enterprises Ltd Edinburgh) as scintillator Recounting of samples after adding a known tritium standard showed that quenching did not occur The average of 39 background measurements was 5 6 cpm (SE = 0 5)

Some of the activity may be insoluble in organic solvents and will therefore remain in the tissues after extraction with chloroform methanol The tissue rests (containing the *insoluble fraction*) were processed by the following method (*Herberg* 1960) The dried samples were washed three times in benzene to remove the last trace of lipid soluble radioactivity 1 5 ml of 1 0 M methanolic Hyamine* hydroxide (Packard) was added and the

* Reg Trade Mark of Rhom & Haas Inc

tissues were completely digested in tightly closed brown vessels in a waterbath at 65° C for 12 hours. Scintillator (PPO POPOP, toluen and ethanol) was added, and the samples were counted in the spectrometer Quenching was evaluated by the method of introducing the automatic external gamma standard into the counting chamber (*Higashimura et al* 1962) The counts recorded were corrected for quenching The average of 42 background measurements was 14 8 cpm (SE = 0,3)

In the lipid-soluble as well as in the insoluble fractions the counts were corrected for background and for differences in counting efficiency and were expressed as counts per minute (cpm) in 1 mg of wet tissue

Thin Layer Chromatography of Tissue Extracts

The degree to which measured tritium was representative of DMBA ^3H was evaluated in separate experiments DMBA ^3H was administered orally to eight mice and intraperitoneally to another eight mice as described above (experiment B-1 and B 2) Two mice from each group were killed after six hours six days and two months and a half Specimens of the liver fat and ovaries were homogenized and processed as described The lipid soluble material was dissolved in 500 µl benzene and 50 µl of this sample was immediately chromatographed and subsequently assayed for radioactivity All operations were performed *in the dark* to prevent degradation of DMBA due to photo toxidation

RESULTS

DMBA ^3H

In two dimensional chromatography of the starting material (*Fig 1*) area eight of the second series (derived *from the fluorescent spot*) represented DMBA (*Boyland & Sims* 1965) *After repeated* two dimensional chromatography of area eight *of the first series* (derived from the application spot) *most of* the counts were refound in area *eight of the* new second series suggesting the presence of DMBA which had not been separated by chromatography in the first *dimension* The total counts contained in areas eight of both series will therefore be interpreted as representative of DMBA while counts contained in areas 1–7 *belong to* substances present in the DMBA ^3H *solution* with other chromatographic properties than DMBA

The distribution of radioactivity after two

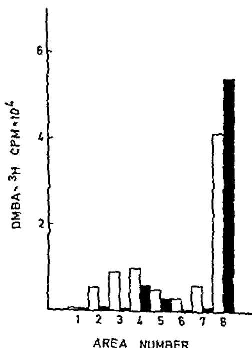


Fig 1 The distribution of radioactivity in two-dimensional chromatography of DMBA-^3H Area eight is closest to the solvent front □ *first series containing the application spot* ■ *second series* containing the fluorescent spot

dimensional chromatography of DMBA ^3H shows that ca 65 per cent should represent DMBA (Fig 1)

Experiment A 1 and A 2 The *results of* oocyte counts appear from Fig 2

In the control ovaries the number of small oocytes decreased exponentially from 6600 at 22 days to about 3200 at 3 *months of age*

After feeding of DMBA a reduction of the small oocytes to 5100 occurred within 24 hours This abrupt decline continued *until 2–3* weeks after treatment Then the rapid decrease of oocytes ceased and the few surviving oocytes were eliminated at a *slower* rate, comparable to that seen in *normal* ovaries

The intraperitoneal application of DMBA caused an even *greater reduction* of the oocyte population within the *first* 24 hours after treatment This very *rapid* decrease of oocytes continued until none were left 1–2 weeks after the treatment

TABLE 1 *Counts per Minute (Mean ± Standard Error)* †

Time after plication	Fat		Ovary		Adrenal	
	Lipidsoluble	Insoluble	Lipidsoluble	Insoluble	Lipidsoluble	Insoluble
6 hours	8728± 847	304±139	6075±2675	189±42	7061±569	207±52
12 „	5670±1148	264±162	2045± 536	176±70	1666±475	279±29
24 „	3193± 335	59± 19	1350± 388	69± 5 0	610±126	136±13
48 „	1545± 65	13± 1,0	422± 103	40± 4,5	262± 49	44±13
72 „	503± 30	10± 1,0	180± 3,0	27± 6 5	215± 18	30± 3 0
5 days	225± 48	6± 3,0	87± 0	22± 2,5	152± 15	23± 2 0
7 „	95± 5,0	6± 1,5	27± 4,6	14± 1,0	105± 2 0	20± 0 5
14 „	31± 12	0	16± 8 1	7± 0	27± 13	4± 0 5
1 month	30± 17	0	9± 2,0	2± 0	23± 9 5	4± 0
2 „	5± 2,0	0	5± 2,0	0	8± 1 5	0
3 „	5± 2,0	0	7± 2,0	0	7± 0	0

Experiment B-1

The tritium activity in the various organs after feeding of DMBA-^{3}H is seen in Table 1 and Fig 3 The highest value in all organs was present already at the first observation 6 hours after feeding, suggesting that maximal absorption from the intestine had been reached by this time The activity in the tissues then decreased exponentially with time

In all tissues—apart from the liver—the highest activity was found in the lipid soluble fraction This fraction also remained in the tissues for a longer period of time than the insoluble one Among the tissues studied the highest activity was found in the fat tissue Somewhat lower counts at comparable levels were recorded in the ovaries and in the adrenals, while muscle and brain only contained little radioactivity The liver differed from other organs in giving a higher tritium count in the insoluble fraction than in the soluble one Very low levels of radioactivity were present in most of the tissues up to 2 or 3 months after ingestion of the DMBA ^{3}H

A unilateral 3 × 3 × 4 mm ovarian tumour was found in one of the 3 month old animals The activities in the tumourous ovary and in its contralateral atrophic ovary were low and of the same size

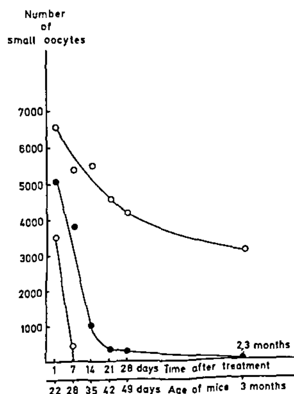


Fig 2 The number of small oocytes in one ovary of control mice ○ *mice treated orally* with DMBA ●, and mice treated intraperitoneally with DMBA ⊙ at the age of 21 days

Experiment B 2

The maximum level of radioactivity after intraperitoneal injection of DMBA-^{3}H (Table 2, Fig 3) was higher in all tissues—except the brain—than after oral administration, and the lipid-soluble activity was considerably higher than the insoluble one in all

m of Various Tissues after Feeding of 150 μCi DMBA ³H

Liver		Muscle		Brain	
p dsoluble	Insoluble	Lip dsoluble	Insoluble	Lipidsoluble	Insoluble
060+259	2060+640	421±90	114+26	417±28	78±13
198+436	1997±574	273±50	97±23	84±24	40± 6 5
270+ 54	1019±103	150±90	54+ 8 0	19+ 1 5	19± 0 5
114+ 21	561± 17	20± 3 0	21± 5 0	7± 0	9± 0,5
63+ 10	480± 79	32±15	19+ 3 0	7± 0	7± 1 5
52 8 5	365± 36	57± 7 0	21± 0 5	7± 0	7± 0 5
26 1 7	156+ 37	10± 7 0	10+ 1 5	5± 2 0	5± 0 5
9 0 9	35+ 4 0	8± 1 5	5± 0 5	3± 0	3± 0
4+ 1 3	10+ 3 0	3± 3 5	4+ 0 5	3+ 0	2± 0 5
0	5+ 2 0	3± 0	3± 3 5	3± 0	1± 0 5
0	3± 0	0	0	0	0

organs apart from the liver The relative distribution of activity was the same as in the feeding experiment with the highest activity in fat lower counts in ovaries and adrenals and still lower counts in liver and muscle

In fat ovaries and adrenals the radio activity increased to reach a maximum 24 hours after the treatment after which time the counts decreased slowly A considerable activity was still present in the last samples investigated 5 months after the intraperitoneal injection The relative behaviour of the lipid soluble and the insoluble fraction in this experiment was similar to that seen in the feeding experiment

3 unilateral ovarian tumours (5 × 5 × 5 mm) were present in 3 and 5 month old animals These tumours gave the same counts as their contralateral atrophic ovaries and the normal sized ovaries of other mice of the same age

As in experiment B I the liver differed from the other organs in that the levels of soluble and insoluble radioactivity were of comparable size (Table 2) The counts in the muscle samples showed marked variations within the first 48 hours of the experiment with large standard errors This is most likely due to inadvertent contamination from peritoneal contents during removal of the femoral muscle which was always the last specimen to be prepared The apparent increase in counts occurring in adrenals towards the end of the experiment is unexplained but could be due to inhomogeneity of the biological material

Droplets of oil could usually be recognized macroscopically on the visceral surface of the peritoneum up to 2 months after intraperitoneal injection of the carcinogen dissolved in olive oil However in 2 animals no traces of oil were seen 24 hours and 5 days after the injection The tissue samples from these animals showed radioactivity at levels comparable to those found after feeding the DMBA ³H It was therefore presumed that the injections accidentally had been given intraintestinally and the results were excluded from the means in Table 2

Chromatography of Tissue Extracts

The distribution of radioactivity from the ovary extracts in different areas of the chromatogram is shown in Fig 4 After oral administration an appreciable amount of radioactivity could only be measured up to 24 hours post treatment most of this activity was located at the site of DMBA (areas eight) In later samples the counts recorded were too small for evaluation.

In the animals treated intraperitoneally the major activity up to day 6 after injection

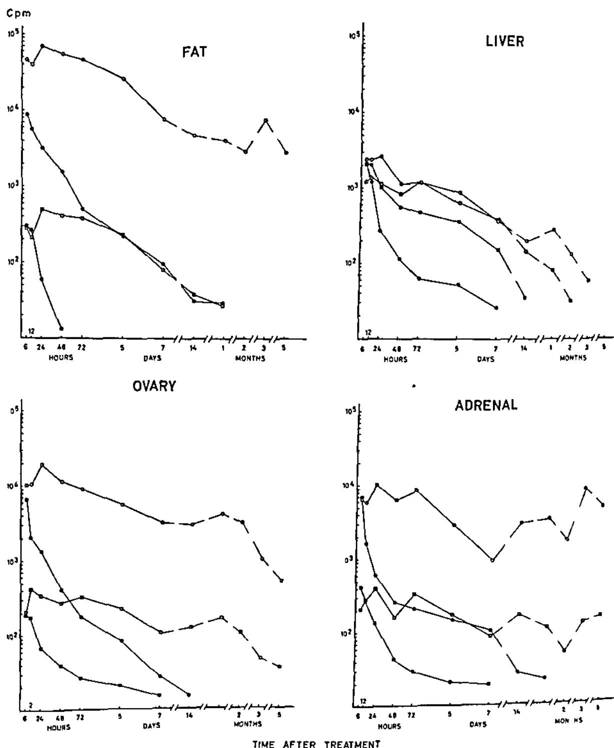


Fig 3 Tritium activity (cpm/mg tissue) in lipid soluble and insoluble fractions of various organs in relation to time after oral or intraperitoneal treatment with 150 μCi DMBA ^{3}H DMBA ^{3}H orally lipid soluble fraction ● insoluble fraction ■ DMBA ^{3}H intraperitoneally lipid soluble fraction ○ insoluble fraction ⊡

was representative of DMBA (areas eight) 2½ months later only few counts were registered In the liver, however, the major activity was still representative of DMBA

DISCUSSION

It is essential in an evaluation of the results to know the extent to which measured radio activity represents DMBA A possible con-

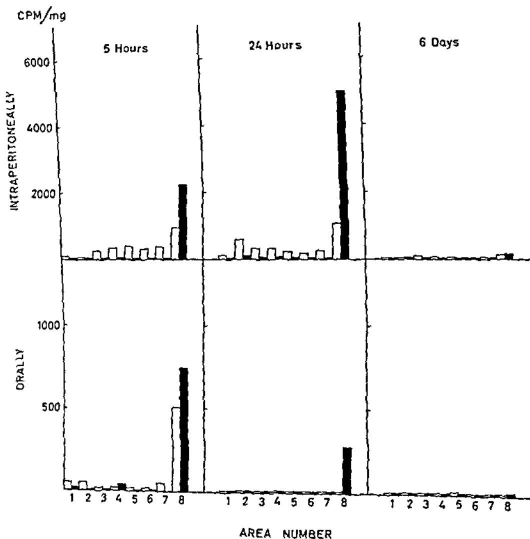


Fig 4 The distribution of radioactivity in two dimensional chromatography of lipid soluble fraction of ovaries in relation to time after oral or intraperitoneal treatment with DMBA-^{3}H Area eight is closest to the solvent front □ first series containing the application spot, ■ second series containing the fluorescent spot

version of DMBA-^{3}H into radioactive intermediary products and a possible separation of ^{3}H from DMBA followed by excretion of tritiated water are factors, which have to be taken into consideration

Two dimensional chromatography of the DMBA ^{3}H in oil solution showed that at least 65 per cent of the radioactivity was representative of DMBA (Fig 1) and this pattern was constant over 9 days This is considerably less than the radiochemical purity of 95 per cent as given by the producer and must be due to storage and preparation under unfavorable conditions This chromatographic pattern (Fig 1) remained largely unaltered in the lipid extracts of ovaries after administration to animals (Fig 4) It is therefore suggested that, at least during the first days after administration, the compound is not changed, and it is therefore unlikely

TABLE 2 *Counts per Minute (Mean ± Standard Error) per M 1 g an*

Time after application	Fat		Ovary		Adrenal	
	Lipidsoluble	Insoluble	Lipidsoluble	Insoluble	Lipidsoluble	Insoluble
6 hours	46007±12599	278±207	10049±3723	209±44	6503±1949	419±86
12	39112±6565	207±40	10803±1444	421±105	58 8±1933	[illegible]±35
24 ,	69871±14696	495±25	19799±3341	348±39	10035±2869	403±193
48	54270±28407	408±161	11389±2094	281±50	6381±1761	163±91
72 ,	46503±996	379±78	9253±1703	346±86	8633±417	339±31
5 days	26215±12348	227±182	5825±729	237±23	2935±115	1 6±64
7	7746±5106	80±15	3344±1581	109±20	956±206	88±15
14	4780±458	38±4 5	3116±605	132±31	3091±478	170±46
1 month	4134±1005	26±11	4396±891	175±27	3495±1350	113±5
2	2916±126	9±9 0	3273±400	110±20	1693±70	50±[illegible]5
3	7697±1393	45±8 0	1066±647	48±22	8525±30[illegible]2	139±[illegible]
5	2848±671	46±16	533±81	36±5 9	4758±768	150±0

that *in vivo* DMBA degradation and the formation of intermediary products are involved in the initiation of ovary carcinogenesis in mice This suggestion is supported by investigations of the carcinogenic power of some DMBA metabolites (*Jull & Russell* 1970) Ovarian tumours arose after treatment with DMBA but not after treatment with the metabolites 7 hydroxymethyl 12 methylbenz(α)anthracene or 7,12 dihydroxymethylbenz(α)anthracene, the carcinogenic power of 12 hydroxymethyl 7 methylbenz(α) anthracene was too weak to accept that the carcinogenic action of the parent DMBA was mediated through this particular metabolite However as in the present study the possibility could not be completely ruled out that the carcinogenic principle was due to oxidation or other chemical change of DMBA in solution already present before administration In experiments with rats *Flesher* (1967) suggested that unchanged DMBA was the active agent in rat mammary carcinogenesis Other studies however demonstrate a role of intermediary products in DMBA carcinogenesis *Boyland & Sims* (1965) showed the formation of two different monohydroxymethylderivatives by rat liver homogenates One of these is capable of inducing mammary tumours and adrenal necrosis in rats (*Boyland et al* 1965) This effect on the adrenal seems to be due to the structural resemblance of the monohydroxymethylderivative to adrenocortical steroids (*Jellinck & Goudy* 1967)

It has been shown (*Daniel et al* 1967) that 20–30 per cent of the isotopic label was released from DMBA ^3H a few hours after ingestion and found as tritiated water in the urine and in the breath For a qualitative evaluation this release of tritium is of minor importance

Experiment B 1 shows that maximal absorption of DMBA from the intestine has been reached within 6 hours after oral ingestion This agrees with earlier experiments DMBA is present in the intestinal lymph 1 hour after ingestion (*Daniel et al* 1967) maximal blood concentration of DMBA is reached within 8 hours (*Hamilton & Jacobson* 1965) and the tissue concentration in rat mammary gland reaches its peak 6 hours after feeding of the substance (*Gammal et al* 1965) After oral administration the unabsorbed DMBA is rapidly excreted in the faeces together with DMBA excreted via the bile after absorption (*Sin Mao* 1965 *Daniel et al* 1967)

Absorption is more prolonged after intraperitoneal injection (experiment B 2) than after feeding as maximal tissue activities were not found until 24 hours after the injection The intraperitoneally deposited DMBA has no possibility of immediate es-

ious Tissues after Intraperitoneal Injection of 150 μCi DMBA ³H

Liver		Muscle		Brain	
Lipidsoluble	Insoluble	Lipidsoluble	Insoluble	Lipidsoluble	Insoluble
2338±316	1206±436	3677±2480	361±214	86± 33	52±36
2336±545	1372±600	743± 130	110± 5,0	151±104	45±25
2599±399	1117± 28	3291±1771	239±169	46± 3,5	17± 0
1128±155	834± 7,5	6845±5752	417±294	30± 10	16± 1,5
1185± 52	1190±160	645± 282	87± 7,0	37± 13	17± 4,0
876±363	633±112	693± 56	53± 16	16± 3,5	9± 1,0
367± 73	388± 93	905± 405	111± 43	10± 0	8± 0,5
202± 28	146± 13	242± 205	31± 5,5	7± 0	4± 1,0
286± 72	82± 10	50± 13	12± 0,5	5± 2,0	3± 0,5
134± 6,5	32± 4,0	101± 1,5	15± 1,0	5± 1,0	2± 0
59± 28	17± 3,0	40± 23	4± 2,0	3± 0	1± 0,5
18± 8,5	26± 9,5	33± 3,5	4± 0,5	3± 0	2± 0,5

cape Oil droplets could be recognized on the peritoneal surface 1 to 2 months after injection and the excretion was not completed 5 months after injection when no activity was present after oral application

A higher concentration of DMBA was recorded in the tissues after intraperitoneal injection than after oral ingestion The absorption from the peritoneum—though slow—must therefore be more complete than from the gut, which may explain the higher tissue concentrations

The general distribution pattern of radioactivity in different tissues found in the present study concurs with that found by other authors Very high concentrations were found in fat tissue (*Flesher* 1967) Fairly high amounts in ovaries and adrenals was also described by *Daniel et al* (1967) and by *Jull & Jellinck* (1968) The low uptake of DMBA by muscle and brain has been observed by *Sin Mao* (1965) and *Daniel et al* 1967

The strains of mice used in the present study develop ovarian tumours after treatment with DMBA (*Krarup* 1967 1970a), whereas adrenal response to the treatment has not been observed On the other hand, rats develop severe adrenocortical necrosis after [illegible]

[illegible] …oplastic ovarian growth [illegible]

(*Jull & Jellinck* 1968) As the tritium uptake after treatment with labelled carcinogen is identical in the adrenals and ovaries of mice (Fig 3, *Jull & Jellinck* 1968) and rats (*Sin Mao* 1965, *Daniel et al* 1967, *Jull & Jellinck* 1968) the different susceptibility of ovaries and adrenals to DMBA in these animals cannot be due to a particularly high ability to concentrate DMBA in the target organs

In an investigation of the uptake of DMBA by mouse ovaries *in vivo* as well as *in vitro* *Jull & Jellinck* (1968) were unable to determine, whether the carcinogenic action was due to the immediate pulse of DMBA passing through the organs or to a residual amount of the substance bound to the ovaries for a much longer time To answer this question two factors have to be considered, *i e* the presence of DMBA in the ovaries in relation to 1) the primary cytological effect (oocyte destruction (*Krarup* 1970b)) and to 2) the ultimate tumour development

The presence of carcinogen in the ovaries correlates well to the destruction of oocytes The destruction of small oocytes when DMBA [illegible] weeks [illegible] with [illegible] is present in the ovaries apart from the very low unchangeable levels found later than 1 month after ingestion (Table 1) The oocyte destruction within the first 24 hours after intraperitoneal

injection of DMBA is larger than after feeding (Fig 2) and is explained by the higher concentration of DMBA in the ovaries within the first 24 hours obtained by this route of application (Table 2) That the depletion of small oocytes becomes total already 2 weeks after intraperitoneal injection of DMBA is due to the maintained high concentration of carcinogen during this period (Table 2)

However, the presence of carcinogen in the ovaries cannot be correlated to the ultimate tumour development Ovarian tumours develop independent on the route of administration (*Krarup 1967, 1970a, Kuwahara 1967*) although DMBA is present in the ovaries for only few weeks after oral ingestion but for at least 5 months after intraperitoneal injection (Fig 3) Furthermore, the ovarian tumours contain no more carcinogen than non-neoplastic ovaries of comparable age (experiments B-1 and B 2), this agrees with observations by *Daniel et al* (1967) who found no radioactivity in mammary tumours 3 months after induction with labelled DMBA The carcinogenic effect of DMBA is therefore probably not due to a prolonged retention of the substance in the ovaries This is consistent with the findings that DMBA induces mammary tumours in rats although it only is present in the target organ for one week or less after ingestion (*Gammal et al* 1965 *Sin-Mao* 1965, *Flesher* 1967) That ovarian tumours develop earlier after intraperitoneal injection of DMBA than after oral ingestion (*Kuwahara* 1967 *Krarup* 1970a) can be explained by the earlier depletion of oocytes in the ovaries of intraperitoneally treated animals as the tumour development is correlated with the onset of oocyte depletion (*Guthrie* 1958 *Krarup* 1970a)

The tentative conclusions which can be drawn from this study are that the carcinogenic effect of DMBA on the mouse ovary most likely is due to the parent hydrocarbon itself rather than to its break down products This effect is exerted immediately after application and consists in destruction of small oocytes which secondarily leads to neoplastic ovarian development, whereas the prolonged retention of DMBA in the ovary after intraperitoneal injection is of no significance for the neoplastic development

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LIPOGRANULOMAS IN HUMAN LIVER BIOPSIES WITH FATTY CHANGE

A Morphological, Biochemical and Clinical Investigation

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The material comprises 67 percutaneous liver biopsies all with fatty change as the chief morphological diagnosis Among others biopsies with cirrhosis and Mallory bodies have been excluded Lipogranulomas have been found in 43 out of 67 biopsies A description of lipogranulomas is given, and three types are described according to size and age Steatosis is a conditio sine qua non for the development of lipogranulomas Biopsies with lipogranulomas show higher histologic activity (lytic liver cell necrosis, acidophilic bodies and mesenchymal reaction) and more parenchymal fibrosis than biopsies without The patients with lipogranulomas have higher S G O T values Whereas lipogranulomas type 1 and 2 can disappear without sequelae there is good reason to believe that lipogranulomas type 3 may give rise to development of connective tissue in the parenchyma

It is a well-known observation that many persons with fatty liver develop fibrosis and eventually cirrhosis, but that in other instances even severe steatosis may be present for years without development of cirrhosis (*Popper 1961*)

Is there a link between steatosis and fibrosis possibly leading to cirrhosis, or is the development of cirrhosis independent of the pre-existing steatosis? It is generally supposed that alcoholic hepatitis is the predominant cause for the development of cirrhosis in alcoholics This is not a sequel to the fatty change in the liver, but is a process that takes place in other liver cells not exhibiting fatty change The possibility that other forms of liver-cell necrosis, e g necroses of fatty liver cells followed by fibrosis, in some cases may be the cause or part of the cause cannot be excluded

We have frequently in liver biopsies with fatty change observed so-called lipogranulomas appearing as isolated or confluent nodular formations consisting predominantly of macrophages (generally lipophages) which as a rule surround one or several extracellular fat vacuoles

The purpose of the work presented here has been to bring lipogranulomas to attention and if possible to obtain particulars of their morphogenesis and to com— — — —ries of

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biopsies both with fatty change as the chief morphological diagnosis, one with and one without lipogranulomas. Furthermore a comparison between a number of biochemical parametres from the corresponding groups of patients as well as a follow up of shorter duration have been performed. However, it has not been possible on the present material to perform a follow up over a longer period of time and such a follow-up is essential for evaluating the possible importance of lipogranulomas for the development of cirrhosis.

MATERIAL AND METHODS

The material consists of a total of 67 percutaneous liver biopsies (from 67 patients), all with fatty change as the chief morphological diagnosis. Biopsies with cirrhosis (defined as nodular regeneration + fibrosis), suspicion of cirrhosis, Mallory bodies, signs of cholestasis of chlorpromazin type or extrahepatic cholestasis, signs of viral hepatitis, malformations, neoplasms and vascular disorders are excluded.

The biopsies have been selected as consecutive primary biopsies from a total of 896 percutaneous liver biopsies received at the Pathological Anatomical Institute, Kommunehospitalet, from medical department B, Bispebjerg Hospital in the period July 1962–November 1968.

The liver biopsies have been performed a.m. Menghini when there was anamnestic or clinical suspicion of liver disease.

The tissue has been fixed in neutral formalin and imbedded in paraffin. From the first biopsies 10–15 sections were cut on a sledge microtome while the last 46 biopsies were cut on a rotory microtome (40–50 sections). The sections are approximately 6 μm in thickness.

The assessment has been performed without knowledge of the *clinical data* by two of the authors (P. C. and O. B.) in close cooperation on haematoxylin and eosin and v. Gieson Hansen preparations. For nearly all biopsies further sections stained for reticulum fibres (*Gomori* 1937), iron (*Perls* 1867) and pyroninophil substance (*Brachet* 1942) have been available.

In six cases lipogranulomas have been examined histochemically for lipids on tissue fixed in Baker's formol calcium (4° C) and after eighteen hours transferred to 0.88 M Acacia sucrose. Serial sections were cut at 7 μm on a SLEE cryostat.

The histochemical reactions performed for lipids were oil red O and Sudan black B (*Lison & Dagnelie* 1935).

Lipogranulomas are quantified in the following manner:

\+ the biopsies contain lipogranulomas, but on an average less than one per lobule,

\+ \+ one or more lipogranulomas per lobule.

In addition to quantitating lipogranulomas a registration of focal lytic liver-cell necroses and acidophil bodies has been performed. Lytic necroses are defined as necroses which appear as defects in the liver-cell plates which are more or less filled in with lymphocytes, plasma cells, and/or proliferating Kupffer cells. Further the degree of fatty change (1–3), the occurrence of Kupffer cell proliferation, cholestasis, and parenchymal inflammation are evaluated. Cholestasis has been registered, when intra- or extracellular bile thrombi have been demonstrated with certainty. In the registration of Kupffer cell proliferation and mesenchymal reaction in general constituents of the lipogranulomas are not included.

Furthermore fibrosis in both portal tracts and parenchyma, bile duct proliferation and portal inflammation and siderosis, as well as occurrence of *lipofuscin* in liver cells and Kupffer cells are registered. As parenchymal fibrosis are registered stellate, smaller or greater zones of collagen fibrils lying in relationship to a central vein or more peripherally in the lobule.

At the time of biopsy the following variables were registered: Alkaline phosphatase (autoanalysator), serum bilirubin (colorimetric), serum asparate transaminase (Reitman & Frankel's method), prothrombin proconvertin (Owren's method), BSP retention (colorimetric), and serum albumin and gamma globulin (paper electrophoresis).

The consumption of alcohol is registered. *Chronic alcoholism* is defined as intake of more than 50 g ethanol/day for more than five years.

Fortyfive rebiopsies have been performed on the 67 patients comprising the material. These biopsies have been assessed according to the same criteria as the primary biopsies. The average period of observation has been 18 months varying from one month to 61 months.

The material is divided into two groups: *Group 1* containing lipogranulomas and *group 2* without. The histologic, biochemical and clinical features in the two groups have been compared.

For the statistical evaluation the chi² test has been used for the histological *parametres, while the t test* has been used for the biochemical variables following logarithmic transformation of the values *for alkaline* phosphatase, serum asparate transaminase and serum gamma globulin (*Dixon* 1965). The limit *for type I error* (2α) has been placed at 0.05.

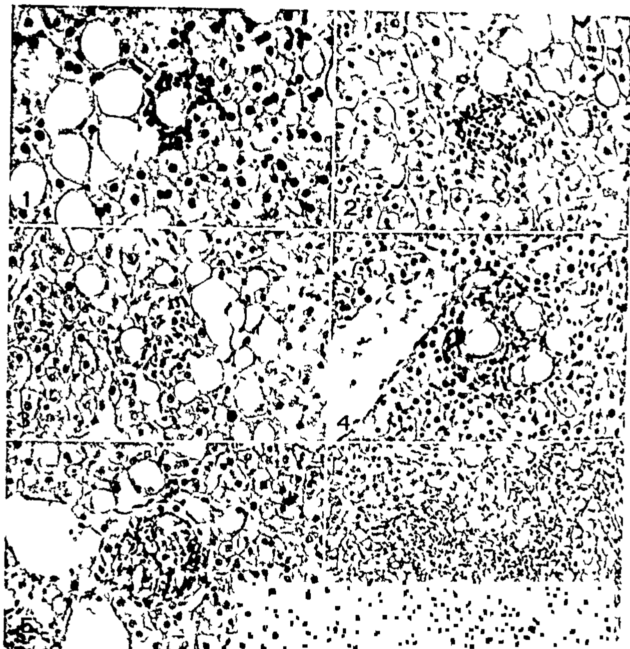


Fig 1 Lipogranuloma type 1 350 ×

Fig 2 Lipogranuloma type 2 with central lipid vacuole 350 ×

Fig 3 Lipogranuloma type 2 without lipid vacuole in this section Serial sections showed central lipid vacuole 350 ×

Fig 4 Lipogranuloma type 3 with many lipid vacuoles 350 ×

Fig 5 Lipogranuloma type 3 with few lipid vacuoles 350 ×

Fig 6 Large lipogranuloma type 3 224 ×

RESULTS

Morphological Findings

Lipogranulomas are found in 43 of the 67 biopsies (group 1) whereas 24 are without lipogranulomas (group 2)

The structure registered as lipogranulomas in this paper present one of the following appearences though it must be noted that there are gradual transitions between the three types

Type 1 Single nodule consisting centrally of a larger extracellular vacuole showing positive staining for lipids and being surrounded by histiocytes (only few of which are of lipophage type) and sometimes lymphocytes and eosinophils (Fig 1)

Type 2 Solitary nodule composed of histiocytes of which the majority are lipophages lymphocytes and sometimes eosinophils and giant cells (Figs 2 and 3) Collagen fibres are few or absent

Serial sections reveal a centrally situated extracellular lipid vacuole

Type 3 Multinodular structure built up of confluent noduli of type 1 and/or type 2 Furthermore there is nearly always a greater or smaller amount of fibroblasts and collagen fibrils in and between the individual nodules (Figs 4 5 and 6)

As all three types of structures are granulomatous and as we are convinced that type 2 develops from type 1 and type 3 consists of confluent type 1 and 2 we feel that it is justifiable to use the term lipogranuloma for them all

Lipogranulomas occur focally singly or in small groups and are encountered in all parts of the lobule where the liver cells contain fat The number of lipogranulomas varies from three to four per biopsy to several per lobule If many lipogranulomas are present they usually are evenly distributed throughout the biopsy though focal accumulations sometimes are encountered Large lipogranulomas type 3 are most frequently found in relation to the central vein

Sometimes lipogranulomas especially the larger assume a pronounced epithelioid cell character and present differential diagnostic problems as opposed to true epithelioid cell granulomas but serial sections reveal one or more lipid vacuoles (*Iversen et al* 1970)

A few biopsies only contain lipogranulomas of either type 1 or type 2 but as a rule lipogranulomas of both type 1 and type 2 are found in the same biopsy Type 3 is demonstrated in four biopsies

In 36 out of the 43 biopsies in group 1 less than one lipogranuloma is found per lobule while the remaining seven biopsies on an average contain more than one and less than three lipogranulomas per lobule

A comparison between most of the histologic features in the two groups is shown in Table 1

Lytic necroses There are a few lytic necroses in the majority of biopsies in both group 1 and 2 but there are with statistical significance more biopsies with lytic necroses in group 1 (42 (98 per cent)) than in group 2 (13 (54 per cent)) ($p < 0.001$)

Acidophilic bodies In group 1 a few acidophil bodies are found in 12 biopsies (28 per cent) whereas they are not found in the biopsies from group 2 There are with statistical significance more biopsies in group 1 with acidophil bodies than in group 2 ($p < 0.05$)

Fatty change There are more biopsies in group 1 with moderate or severe fatty change (25 (59 per cent)) than in group 2 (eight (33 per cent)) but the difference is not statistically significant ($p > 0.05$)

Kupffer cell proliferation Kupffer cell proliferation is exhibited by all biopsies in group 1 while five (21 per cent), are without Kupffer cell proliferation in group 2 The difference is significant ($p < 0.05$)

Parenchymal inflammation There are more biopsies with inflammation of the parenchyma in group 1 (15 (35 per cent)) than in group 2 (three (13 per cent)) This difference is not statistically significant ($p > 0.10$) The inflammatory infiltrate in all cases contains lymphocytes while eosinophils only are demonstrated in one biopsy from group 1

Parenchymal fibrosis There are more biopsies with parenchymal fibrosis in group 1 (13 (30 per cent)) than in group 2 (three (13 per cent)) This difference is not statistically significant ($p > 0.10$) The four biopsies with lipogranulomas type 3 all exhibit parenchymal fibrosis whereas only nine

TABLE 1 *The Frequency in the Material of a Series of Histological Qualities in Group*

	Lytic liver cell necroses		Acidophilic bodies		Degree of fatty infiltration		
	0	+	0	+	+	++	+++
Group 1 (43 biopsies with lipogranulomas)	1 (2 %)	42 (98 %)	31 (72 %)	12 (28 %)	18 (42 %)	17 (40 %)	8 (19 %)
Group 2 (24 biopsies without liopgranulomas)	11 (46 %)	13 (54 %)	24 (100 %)	0 (0 %)	16 (67 %)	7 (29 %)	1 (4 %)
Differences (χ^2 test)	$p < 0.001$		$p < 0.05$		$0.05 < p < 0.10$		

The figures indicate the number of biopsies in each subgroup of the seven qualities The percentage the number comprises of the total subgroup is given in brachets

of the 39 biopsies with lipogranulomas 1 and/or 2 show similar fibrosis

Portal inflammation There are more biopsies in group 1 (31 (72 per cent)) with portal inflammation than in group 2 (12 (50 per cent)) but the difference is not significant ($p > 0.10$)

Statistically there is no significant difference between the two groups as regards the incidence of portal fibrosis bile duct proliferation cholestasis siderosis and content of lipofuscin

Scarcely half of the total number of biopsies in both groups exhibit portal fibrosis

Bile duct proliferation has only been demonstrated in few biopsies and only one biopsy (in group 1) exhibits cholestasis

All biopsies contains lipofuscin and approximately one third (from both groups) exhibit siderosis

In summary it may be concluded that there are significantly greater incidence of lytic liver cell necroses acidophil bodies and Kupffer cell proliferation in group 1 than in group 2 while there is no certain difference in the degree of fatty change or in the incidence of parenchymal and portal inflammation parenchymal and portal fibrosis bile duct proliferation cholestasis siderosis or content of lipofuscin It should be mentioned that all four biopsies from group 1 with lipogranulomas 3 exhibit parenchymal fibrosis

Biochemical Findings and Clinical Findings

The distribution of the laboratory results in group 1 and group 2 is given in Table 2 Serum aspartate transaminase is higher with statistical significance ($p < 0.001$) in the group with lipogranulomas as is also seen from Fig 7

Bromsulphalein (BSP) retention is elevated in both groups and there is no difference The other biochemical variables lie inside—or on the border line of—the normal range

The distribution according to sex and age shows no difference between group 1 and 2 The average age for the entire material is 65 years (range 37–83 years) and 70 per cent are men

31 (73 per cent) of the 43 biopsies in group 1 and 17 (69 per cent) of the 24 biopsies in group 2 are from patients stated to be chronic alcoholics There is no significant difference in the incidence of known chronic alcoholism in the two groups

Follow up From 34 of the 67 patients comprising the material are there one or more repeat biopsies (25 patients one seven two and two three) The incidence of lipogranulomas and parenchymal fibrosis in the

3 Biopsies with Lipogranulomas) and Group 2 (24 Biopsies Without Lipogranulomas)

Kupffer cell proliferation		Parenchymal inflammation		Parenchymal fibrosis		Portal inflammation	
0	+	0	+	0	+	0	+
0 (0 %)	43 (100 %)	28 (65 %)	15 (35 %)	30 (70 %)	13 (30 %)	12 (28 %)	31 (72 %)
5 (21 %)	19 (79 %)	21 (88 %)	3 (13 %)	21 (88 %)	3 (13 %)	12 (50 %)	12 (50 %)
$p < 0\ 05$		$0\ 10 < p < 0\ 20$		$0\ 10 < p < 0\ 20$		$0\ 10 < p < 0\ 20$	

biopsies from these 34 patients appears from Table 3

In 19 patients the first biopsy contains lipogranulomas of type 1 and/or type 2 (none with type 3)

Further it appears that lipogranulomas are still present in repeat biopsies from 13 patients (three with type 3), while it has not been possible to demonstrate lipogranulomas in later biopsies from six patients

Out of the total of 15 patients without lipogranulomas in the first biopsy there are

TABLE 2 *The Distribution of the Biochemical Variables at the Time of Biopsy in the Group With Lipogranulomas (Group 1) and in the Group Without Lipogranulomas (Group 2)*

Laboratory test (Normal value)	Group 1 + lipogranulomas mean ± s d	Group 2 no lipogranulomas mean ± s d	Significance (t test)
Alkaline phosphatase (< 10 King Armstrong units)	11 5 ± 5 0	10 0 ± 5 4	no significance*
Serum bilirubin (< 1 0 mg/100 ml	1 0 ± 0 8	0 9 ± 0 6	no significance
Serum asparate transaminase (< 1 7 mmol/l/hr)	3 3 ± 1 7	1 9 ± 0 8	$P < 0\ 001$*
Prothrombin proconvertin (> 60 % of normal)	83 ± 13	79 ± 15	no significance
BSP retention (< 5 % after 45 min)	14 ± 9	11 ± 7	no significance
Serum albumin (> 4 4 g/100 ml	4 6 ± 0 87	4 72 ± 0 70	no significance
Serum γ-globulin (< 1 1 g/100 ml	1 14 ± 0 39	0 97 ± 0 43	no significance*
Total number	43	24	

* After logarithmic transformation

TABLE 1 *The Frequency in the Material of a Series of Histological Qualities in Group*

	Lytic liver cell necroses		Acidophilic bodies		Degree of fatty infiltration		
	0	+	0	+	+	++	+++
Group 1 (43 biopsies with lipogranulomas)	1 (2 %)	42 (98 %)	31 (72 %)	12 (28 %)	18 (42 %)	17 (40 %)	8 (19 %)
Group 2 (24 biopsies without lipogranulomas)	11 (46 %)	13 (54 %)	24 (100 %)	0 (0 %)	16 (67 %)	7 (29 %)	1 (4 %)
Differences (χ^2 test)	$p < 0.001$		$p < 0.05$		$0.05 < p < 0.10$		

The figures indicate the number of biopsies in each subgroup of the seven qualities. The percentage the number comprises of the total subgroup is given in brackets.

of the 39 biopsies with lipogranulomas 1 and/or 2 show similar fibrosis.

Portal inflammation. There are more biopsies in group 1 (31 (72 per cent)) with portal inflammation than in group 2 (12 (50 per cent)) but the difference is not significant ($p > 0.10$).

Statistically there is no significant difference between the two groups as regards the incidence of portal fibrosis, bile duct proliferation, cholestasis, siderosis and content of lipofuscin.

Scarcely half of the total number of biopsies in both groups exhibit portal fibrosis.

Bile duct proliferation has only been demonstrated in few biopsies and only one biopsy (in group 1) exhibits cholestasis.

All biopsies contain lipofuscin and approximately one third (from both groups) exhibit siderosis.

In summary it may be concluded that there are significantly greater incidence of lytic liver cell necroses, acidophil bodies and Kupffer cell proliferation in group 1 than in group 2, while there is no certain difference in the degree of fatty change or in the incidence of parenchymal and portal inflammation, parenchymal and portal fibrosis, bile duct proliferation, cholestasis, siderosis or content of lipofuscin. It should be mentioned that all four biopsies from group 1 with lipogranulomas 3 exhibit parenchymal fibrosis.

Biochemical Findings and Clinical Findings

The distribution of the laboratory results in group 1 and group 2 is given in Table 2. Serum asparate transaminase is higher with statistical significance ($p < 0.001$) in the group with lipogranulomas, as is also seen from Fig. 7.

Bromsulphalein (BSP) retention is elevated in both groups, and there is no difference. The other biochemical variables lie inside—or on the border line of—the normal range.

The distribution according to sex and age shows no difference between group 1 and 2. The average age for the entire material is 65 years (range 37–83 years), and 70 per cent are men.

31 (73 per cent) of the 43 biopsies in group 1 and 17 (69 per cent) of the 24 biopsies in group 2 are from patients stated to be chronic alcoholics. There is no significant difference in the incidence of known chronic alcoholism in the two groups.

Follow up. From 34 of the 67 patients comprising the material are there one or more repeat biopsies (25 patients one, seven two and two three). The incidence of lipogranulomas and parenchymal fibrosis in the

3 Biopsies with Lipogranulomas) and Group 2 (24 Biopsies Without Lipogranulomas)

Kupffer cell proliferation		Parenchymal inflammation		Parenchymal fibrosis		Portal inflammation	
0	+	0	+	0	+	0	+
0 (0 %)	43 (100 %)	28 (65 %)	15 (35 %)	30 (70 %)	13 (30 %)	12 (28 %)	31 (72 %)
5 (21 %)	19 (79 %)	21 (88 %)	3 (13 %)	21 (88 %)	3 (13 %)	12 (50 %)	12 (50 %)
$p < 0.05$		$0.10 < p < 0.20$		$0.10 < p < 0.20$		$0.10 < p < 0.20$	

biopsies from these 34 patients appears from Table 3

In 19 patients the first biopsy contains lipogranulomas of type 1 *and/or type 2* (none with type 3)

Further it appears that lipogranulomas are still present in repeat biopsies from 13 patients (three with type 3), while it has not been possible to demonstrate lipogranulomas *in later biopsies* from six patients

Out of the total of 15 patients without lipogranulomas in the first biopsy there are

TABLE 2 *The Distribution of the Biochemical Variables at the Time of Biopsy in the Group With Lipogranulomas (Group 1) and in the Group Without Lipogranulomas (Group 2)*

Laboratory test (Normal value)	Group 1 + lipogranulomas mean ± s d	Group 2 no lipogranulomas mean ± s d	Significance (t test)
Alkaline phosphatase (< 10 King Armstrong units)	11.5 ± 5.0	10.0 ± 5.4	no significance*
Serum bilirubin (< 1.0 mg/100 ml)	1.0 ± 0.8	0.9 ± 0.6	no significance
Serum asparate transaminase (< 1.7 mmol/l/hr)	3.3 ± 1.7	1.9 ± 0.8	$P < 0.001$*
Prothrombin-proconvertin (> 60 % of normal)	83 ± 13	79 ± 15	no significance
BSP retention (< 5 % after 45 min)	14 ± 9	11 ± 7	no significance
Serum albumin (> 4.4 g/100 ml)	4.6 ± 0.87	4.72 ± 0.70	no significance
Serum γ globulin (< 1.1 g/100 ml)	1.14 ± 0.39	0.97 ± 0.43	no significance*
Total number	43	24	

* After logaritmic transformation

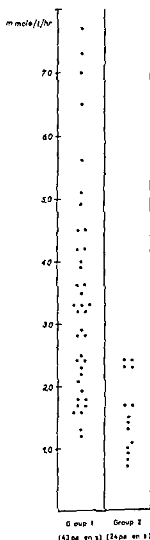


Fig 7 The distribution of serum asparate transaminase values in the group with lipogranulomas (group 1) and in the group without lipogranulomas (group 2)

still none in the repeat biopsies from seven patients while lipogranulomas now are present in biopsies from eight patients

From Table 3 it further appears that parenchymal fibrosis has only been found in biopsies with lipogranulomas The figures are very small However, it may be mentioned that the fibrosis in the majority of cases was unchanged from the first to the later biopsies but it may be both increased and decreased

DISCUSSION

The structure designated as *lipogranuloma type 1* in this paper has previously been described by inter alii *Leevy et al* (1953) *Hartroft* (1954), and *Popper* (1961) and have by these been called large fatty cyst "Eventually with prolonged steatosis fat drops of several neighbouring cells coalesce to a large fatty cyst which now lies extracellularly, producing an inflammatory reaction which consisted of lymphocytes and an exudate" (*Leevy et al* 1953)

Our view on the morphogenesis of this structure corresponds with the quoted and is supported by the following circumstances 1) lipogranulomas are always encountered in areas where the liver cells contain fat and the surrounding liver cells frequently contain very large lipid vacuoles and 2) the transition between the liver cell with fatty change on the one hand and the lipogranuloma on the other hand is sometimes seen as a ruptured liver cell with fat deposited both intracellularly and extracellularly

While *Hartroft* (1954) assigns the fatty cysts importance by compromising the blood supply to the central part of the lobule and thereby provoking central necrosis *Leevy et al* (1953), and later *Thaler* (1969) who described the same structure under the name of 'Resorptionsknötchen' are of the opinion

TABLE 3 *The Incidence of Lipogranulomas and Parenchymal Fibrosis in the Primary Biopsies and the Rebiopsies from 34 Patients*

	1 biopsy	rebiopsies
34 patients	19 pts lipogranulomas (7 fibrosis)	13 pts ÷lipogranulomas (6 fibrosis)
		6 pts ÷lipogranulomas (fibrosis)
	15 pts lipogranulomas (÷fibrosis)	8 pts lipogranulomas (3 fibrosis)
		7 pts ÷lipogranulomas (÷fibrosis)

that this is a transient structure without further importance (*Thaler* 1969)

In conformity with *Leevy et al* (1953) and *Thaler* (1969) we regard lipogranuloma type 1 as transient changing into lipogranuloma type 2 This opinion is supported by transitory forms between type 1 and type 2 often being found in the biopsies

Lipogranuloma type 2 has previously been described by *Popper* (1961) as lipogranuloma and by *Thaler* (1969) as 'Resorptionsknötchen It is *Thaler's* (1969) opinion that this structure disappears, and it is also our experience from repeat biopsies that both the lipophages and any lymphocyte infiltration can disappear without any sequelae

Lipogranuloma type 3 Thaler (1969) reported three cases of granulomatous hepatitis of unknown type in livers with fatty change Micrographs and descriptions are in accordance with our lipogranuloma type 3 Apart from these three cases, this type has not so far as the authors are informed, previously been described in human liver tissue

We do not regard this structure as a further development of an isolated lipogranuloma type 1 or type 2 but as the result of many confluent lipogranulomas type 1 and type 2 This is best seen in serial sections where the nodular structure of lipogranuloma type 3 is clearly seen While it is the rule that lipogranuloma type 1 and type 2 disappear without any sequels we do not believe that this is the case with lipogranuloma type 3 as we, as a rule find fibrosis in the biopsies with lipogranulomas type 3 not only in the lipogranulomas but also in other parts of the parenchyma mostly centrilobularly

Differential diagnosis Lipogranulomas have in general a typical appearance, but can in rare cases where the picture is dominated by epithelioid-cell like lipophages in many of the serial sections be impossible or nearly impossible to differentiate from actual epithelioid cell granulomas The most reliable way to separate the lipogranulomas from the latter is the use of serial sections to demonstrate fat vacuoles A suspicion of the real nature of the granulomas may, however be obtained, as they—contrary to the epithelioid cell granulomas—only are found in the parenchyma, frequently in close relationship to a central vein, and by these very frequently being accompanied by more typical lipogranulomas in *other* parts of the biopsy (*Iversen et al* 1970)

Occurrence Lipogranulomas type 1 and 2 must be considered as occurring very frequently in livers with fatty change, while lipogranulomas type 3 are rather infrequently encountered

Activity If the histological activity in a biopsy is expressed by the number of necrotic liver cells there is in our material a greater activity in the group of biopsies with lipogranulomas than in the group without, even if the lipogranulomas are disregarded

The biochemical activity is also greater in the group with lipogranulomas where S G O T is greater with statistical significance How much of the S G O T—elevation is due to lytic liver-cell necroses and how much is due to lipogranulomas cannot be decided on the available facts

Do the lipogranulomas directly determine the increased number of lytic necroses and acidophil bodies, or is the formation of lipogranulomas, lytic necroses, and acidophil bodies parallel, more or less independent processes?

The condition, that the lytic necroses and the acidophil bodies nearly always are spatially distinct (proved by going through serial sections) and therefore nearly never lie in intimate contact with lipogranulomas, speaks against the lipogranulomas themselves inducing the development of lytic necroses and acidophil bodies As a considerable degree of positive correlation is, however, found between the incidence of all three kinds of liver-cell disintegration, it may with certainty be said, that there is a connection in time, and it is probable that there is an aetiological connection as well

The activity one finds in liver tissue with steatosis in form of lytic necroses and acidophil bodies is by *Popper* (1961) and *Thaler* (1969) considered to be a parallel effect of

the agent causing steatosis (alcohol, malnutrition or intoxication) and is not thought to be determined by the fatty change This view is compatible with the present results It is, however, our opinion that the part of the morphological activity which is due to the lipogranulomas is a result of the steatosis, and that fatty change is a conditio sine qua non for the development of the lipogranulomas

It can in this connection be stated that lipogranulomas most frequently, but not exclusively, are to be found in alcoholics and conversely, only about half of the alcoholics exhibiting fatty change have at the same time lipogranulomas

Does the increased activity have any consequence for the development of fibrosis and/or cirrhosis? Most observers call for a link between steatosis and fibrosis eventually leading to cirrhosis As such a link hepatic necrosis has been suggested (*Popper* 1961) We have in the present material found disintegration of liver cells in the form of lytic necroses acidophil bodies and lipogranulomas

On follow up it has been shown that some of the patients with lipogranulomas lytic necroses and acidophil bodies in the first biopsy are without these in repeat biopsies In these cases the lipogranulomas in question have only been of type 1 and type 2 Further it is noteworthy that—in spite of some cases exhibiting slight parenchymal fibrosis in the first biopsy—there is no fibrosis in the repeat biopsies

In accordance with this it must be permissable to conclude that lytic necroses acidophil bodies and lipogranulomas type 1 and 2 usually disappear without sequelae and can therefore not be a link between steatosis and fibrosis Further the findings indicate that slighter fibrosis may disappear

Lipogranulomas type 3 on the other hand show a tendency to fibrosis with transitions from lipogranulomas with fine collagen fibres to lipogranulomas with a dense collagen connective tissue containing a few lipophages Our investigation further shows that there is more pronounced parenchymal fibrosis with larger, often stellate areas of connective tissue in biopsies with lipogranulomas type 3 than in the others As furthermore the parenchymal fibrosis in by far the greatest majority of cases is situated centrilobularly and with the same localization as lipogranulomas type 3 this affords good grounds for believing that the big lipogranulomas may give rise to development of connective tissue in the parenchyma A follow up examination of patients with lipogranulomas type 3 is not available and is necessary for an estimation of the possible significance of lipogranulomas type 3 for the development of cirrhosis

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Our thanks are due to *H Lyon* M D Dept. of Path Anat Kommunehospitalet for evaluation of histochemical data

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HETEROTRANSPLANTATION OF HUMAN ADENOCARCINOMAS OF THE COLON AND RECTUM TO THE MOUSE MUTANT NUDE. A STUDY OF NINE CONSECUTIVE TRANSPLANTATIONS

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Pathological Anatomical Department, Kommunehospitalet, Copenhagen
Heads H Poulsen and P Christoffersen

Successful heterotransplantation of 6 out of 9 consecutively transplanted adenocarcinomas of the human colon and rectum to the mouse mutant Nude is reported Serial growth for 2 8 transfers so far was obtained in 5 cases The transferred tumours grow locally and metastatic growth has not been observed with certainty The histological and cytological appearance of the transferred tumours is in full accordance with that of the primary tumours The mutant Nude is suggested as a host for human malignant tumours as a basis for the study of tumour growth and for therapeutic assays

Heterotransplantation of a human malignant tumour to the mouse mutant Nude, suffering from recessive thymic aplasia has been described previously (5) The purpose of this paper is a further examination of the growth pattern of human carcinomas of the colon and rectum in this mutant as demonstrated in nine consecutive heterotransplantations

MATERIALS AND METHODS

Six to ten week old mice of the mutant Nude bred in Pathological Anatomical Department, Kommunehospitalet Copenhagen were employed Controls in the primary transfers were phenotypically normal littermates presenting a normal fur They were inoculated in the same manner as the Nude mice to exclude a loss of antigenicity in the primary tumour For the primary transfers 3 6 Nude mice and one control were inoculated The following transfers were performed in the same number of Nude mice and in controls as far as the amount of tumour tissue allowed The tumour tissues were taken from patients undergoing abdominal surgery in Surgical Department 1, Kommunehospitalet, Copenhagen The tumours were chosen randomly within a period of three months

The interval between removal and implantation of the tumours has varied from 15 to 45 minutes Either solid tumour blocks measuring 2 × 2 × 2 mm or 0 5 ml of a suspension of tumour material in tissue culture medium were used as inocula as formerly described (5) The inocula were implanted subcutaneously in the lateral abdominal wall

The same methods were used in the following transfers, except for 5 randomly chosen cases which were injected intraperitoneally

Patient data localization and spread of tumour, histology and details of methods of primary inoculations are given in Table 1

Primary human tumours have been subject to routine histological examination at the Pathological

Received 8 x 70

Requests for reprints should be adressed to C O Povlsen, Pathological Anatomical Department, Kommunehospitalet, 1399 Copenhagen, Denmark

TABLE 1 *Patient Data, Localization and Spread of T*

Case number	Clinical data	Type of operation	Size localization and spread of tumour
1	72 year ♂ (20106) 2–3 weeks abdominal pain and obstipation Considerable weight loss	10 10 69 lap c *resect recti*	Tumour of the rectum of the size of a fist Metastases in liver and mesentery
2 *	74 year ♀ (20246) 2 months of obstipation and diarrhoea	7 10 69 lap c *resect* coli sigmoid	Circular 3 cm broad tumour of sigmoid colon with total penetration of the wall Several big metastases of the liver
3	87 year ♀ (20789) 3 weeks of severe obstipation *Weight loss* 25 kg in 1 year Slight periodical abdominal pain	30 12 69 lap c ileotransverso stomia	Double fist big tumour of the transverse colon adherent to *anterior* abdominal wall duodenum and mesentery Several big metastases in liver and mesentery
4	70 year ♂ (20095) A few weeks obstipation Admitted with ileus	8 1 70 *lap* c *hemicolec* tomia dxt	4×6×7 cm tumour of transverse colon with broad penetration of the wall Peritoneal carcinosis and ascites Several metastases in liver and mesentery
5	41 year ♀ (20752) *For several* years colitis with obstipation and diarrhoea During 2 months abdominal pain with melaena and fresh blood on stools periodically	13 1 70 lap c resect coli sigmoidei	1 5×4×4 cm tumour of sigmoid colon with total penetration *Metastases in mesenteric lymphnodes*
6	61 year ♀ (20956) Obstipation for several years *Admitted with ileus*	31 12 69 colo *stomia* 26 1 70 lap c resect coli sigmoid	5 cm long 1 cm thick stricturating tumour of sigmoid colon with total penetration
7	67 year ♀ (20961) During 3 months diarrhoea of dark colour *In the same period anorexia and* loss of weight 4 5 kg	26 1 70 lap c *hemicolectomia* dxt et resect duodeni	4½ cm long tumour of the transverse colon with total penetration of the wall and several metast of the mesentery

stology and Details of Methods of Primary Inoculations

Postoperative course	Histological diagnosis	Origin of inoculum	Time before inoculation (minutes)	Inoculation method	Take	Number of transfers
ed 25 days after operation atopsy showed metastases of ng and liver	Adeno carcinoma recti KH 6925/69	Serosal side of primary tumour	45	Tumour cellsusp s c	0	
ncomplicated Dismissed 13 days er operation Readmitted 4 70 Died 35 days later atopsy showed carcinosis ritonei and enormous metastases liver	Adeno-carcinoma colloides coli KH 6792/69	Serosal side of primary tumour	15	Tumour cellsusp s c	+	8
ed 10 days after operation topsy not performed	Adeno-carcinoma coli KH 8917/69	Metastases of mesentery of small intestine	20	Tumour cellsusp s c.	0	
ismissed 14 days after peration Died 10/5 70 atopsy showed carcinosis ritonei and metastases of liver	Adeno-carcinoma coli KH 197/70	Serosal side of primary tumour	45	Tumour cellsusp s c	0	
smissed on day 13 after eration live 1 9 1970	Adeno carcinoma coli KH 281/70	Serosal side of primary tumour	35	Tumour cellsusp s c	+	2
ismissed 4 days after operation live 1 9 1970	Adeno carcinoma coli KH 605/70	Serosal side of primary tumour	30	Tumour blocks s c	+	3
ismissed 4 days after operation live 1 9 1970	Adeno carcinoma partim anaplast coli KH 629/70	Serosal side of primary tumour	30	Tumour blocks s c	+	7

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ı missed on day 13 after eration. live 1 9 1970	Adeno carcinoma coli KH 281/70	Serosal side of primary tumour	35	Tumour cellsusp s c	+	2
ısmissed ł days after operation. live 1 9 1970	Adeno-carcinoma coli KH 605/70	Serosal side of primary tumour	30	Tumour blocks s c	+	3
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7	67-year ♀ (20961) During 3 months diarrhoea of dark colour In the same period anorexia and loss of weight 4–5 kg	26 1 70 lap c hemicolectomia dxt *et resect* duodeni	4½ cm long tumour of the transverse colon with total penetration of the wall and several metast of the mesentery

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Postoperative course	Histological diagnosis	Origin of inoculum	Time before inoculation (minutes)	Inoculation method	Take	Number of transfers
mis ed da after operation e 1 9 1970	Adeno carcinoma coli KH 906/70	Serosal side of primary tumour	30	Tumour blocks s c	+	1
d 10 days after operation autopsy	Adeno carcinoma KH 1023/70	Serosal side of primary tumour	40	Tumour blocks s c	+	2

scribed apparently encapsulated, solid masses The bigger tumours bulge into the abdominal cavity displacing the muscles of the abdominal wall and lifting the parietal peritoneum which seems intact over the tumour Relatively large vessels are seen crossing and penetrating the tumours. The cut surfaces are greyish white, finely granulated with yellow necrotic areas The appearance of case no 2 differs from the above mentioned the cut surface being dominated by mucoid material

In three instances (transfers of case no 2) following intraperitoneal inoculation large tumour masses were found in the mesentery of the small intestine and in two instances out of these growth was located to the surface of the liver No metastases in the draining lymphnodes have been observed following subcutaneous inoculation In no case ascites has developed No metastases to the lungs have been observed

The life span of the hosts does not seem to be influenced as compared to untreated Nude mutants They generally live 4–5 months (2 3 months after tumour grafting) and the cause of death of both untreated and tumour grafted mice is that of wasting with no uniform pattern

Microscopical findings (primary and transfers) In all cases histological examination of the operative specimens of primary human tumours has confirmed that there is penetration of the colon wall In transfers tumour growth has macroscopically appeared to occur in the space between panniculus musculosus and the muscles of the abdominal wall This localization has also been confirmed on the microscopical examination (Fig 2) There may be some flattening and even disappearance of the muscular layers both superficially and profound to the tumours In cases where the abdominal muscles have disappeared or been dislocated, the parietal peritoneum is found intact Tumours appear circumscript, but not encapsulated

Only in a few cases small tumour islands have been seen around the main tumour, always in close relationship to the latter (Fig 3 b) In the periphery of all tumours thus walled, dilated blood vessels are numerous, but only few vessels are seen in the tumour tissue itself In no case invasion of vessels has been observed with certainty Cellular inflammatory infiltration of primary tumours shows great variation but in transferred tumours there is only scarce infiltration, mainly with granulocytes and large mononucleated cells

Microscopical examination of lymphnodes

Case number	Clinical data	Type of operation	Size, localization and spread of tumour
8	66 year ♂ (21001) 8 months before admission fresh blood per rectum 4 months of obstipation Weight loss of 4 kg during last 2 months	5 2 70 lap c resect coli palliativa	3×5×5 cm tumour of the sigmoid colon with total penetration of the wall Several big metastases of the liver
9	66-year ♀ (20936) Weight loss of 10 kg in 2 months 2 weeks of severe obstipation and abdominal pain	17 1 70 coecostomia 10 2 70 lap c hemicolectomia sin	5 cm long stricturating tumour of the sigmoid colon with penetration of the wall and invasion of the anterior abdominal wall Metastases of the liver and ascites

* The first two passages of this tumour have been described previously (5) The tumour is included in this series for two reasons 1) it has been maintained for more transfers than any other tumour in this study, and 2) it has as the only tumour so far given rise to changes that could be interpreted as metastases following intraperitoneal inoculation

Anatomical Department and the slides have been reexamined for comparison with transferred tumours as described later

The mice have been observed and biopsies have been taken at various intervals following inoculation All mice inoculated have later undergone autopsy

Specimens for histological examination have been fixed in formalin, imbedded in paraffin, sectioned at 7 μm and stained with H & E, van Gieson-Hansen, the Alcian blue method (4), and methyl green-pyronin method (4)

RESULTS

Growth has been obtained in six out of a total of nine transferred tumours (cases no 2, 5, 6, 7, 8, 9) The number of transfers with take in the different tumours are seen from Table 1 Tumours have in no case shown take in the controls

The rate of take in the different transfers has roughly been 50 per cent, varying from 0–100 per cent

The pattern of macroscopic growth of the various tumours in all transfers has been very uniform and the cases will be described together Tumour growth appears as a slowly growing nodule at the site of inoculation Tumour growth can be recognized between 10 and 14 days following inoculation The tumours increase slowly in size and may reach considerable dimensions (Fig 1) The biggest tumour has thus measured 25×25×25 mm



Fig 1 Case no 7, transfer no 7 58 days after inoculation

Macroscopic appearance The fully developed tumours are grossly lobulated The overlying skin is generally intact and not adherent, but may ulcerate, probably due to mechanical injury On section the tumours are seen growing in the subcutaneous space as well-circum-


3a
3b

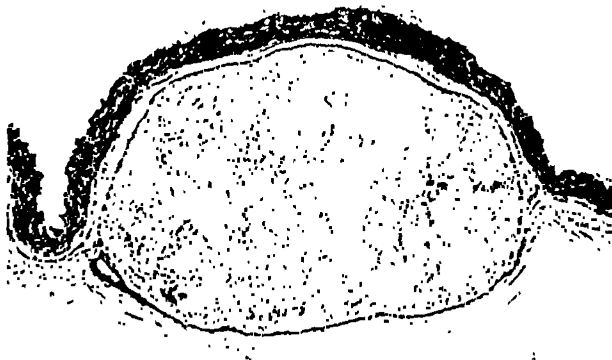


Fig 2 Microscopic appearance of tumour (case no 7, transfer no 3) in the subcutaneous space between the skin and the muscular layer of the abdominal wall, which appears somewhat flattened Two vessels are seen at the left angle of the tumour. 16 × van Gieson-Hansen

and lungs has not shown tumour growth in any case

In the following individual primary tumours and corresponding transferred tumours are described microscopically according to the histological and cytological classifications used by *Evans* (1)

Case 1

Primary tumour Poorly differentiated adenocarcinoma. Cytologically pleomorphic with several mitoses The stromal component is poorly developed Slight infiltration with granulocytes, lymphocytes, and plasma cells Regional lymphnode shows extensive tumour growth

Transfer no take

Case 2

Primary tumour Well differentiated adenocarcinoma The epithelium is rather uniform, predominantly columnar, but in some areas with cuboidal flattening Abundant production of mucin High number of mitoses with several atypical forms Sparce stromal component with moderate infiltration with granulocytes and lymphocytes (Fig 3 a)

Transfers· The histological appearance of the tumour tissue of all transfers is grossly identical Adenomatous formations of the same type as those of the primary tumour are found throughout (Figs 3 a and 3 b). In the bigger tumours there is a dominance of confluent mucoid areas, giving a cystic appear-

Fig 3 a Section from operative specimen (case no 2, KH 6792/69) 56 × H & E

Fig 3 b Case no 2, transfer no 1 The histological picture is the same as in Fig 3 a A small tumour island is seen in the muscular layer The parietal peritoneum (at arrow) is intact 40 × H & E

Fig 4 a Section from operative specimen (case no 6, KH 605/70) Smaller and larger adenomatous formations separated by abundant stroma 160 × *H & E*

Fig 4 b Case no 6, transfer no 2 More solid epithelial structure than seen in Fig 4 a The adenomatous formations are very similar and the main difference is a loss of stroma 160 × H & E



Fig 5 a Section from operative specimen (case no 7 KH 629/70) Anaplastic part of the tumour 400 × H & E

Fig 5 b Case no 7 transfer no 3 Anaplastic tumour tissue from the margin of the tumour A few layers of connective tissue are separating the tumour cells from the muscular layer of the abdominal wall 400 × H & E

Fig 6 Case no 5, transfer no 1 Histologically vital tumour tissue at the margin with extensive necroses towards the centre 160 × H & E

ance. Various degrees of necrosis are observed mainly in the central parts of the tumours. The stroma is poorly developed and there are only a few infiltrating neutrophil granulocytes and large mononucleated cells. Cytologically there is an almost complete correspondance with the primary tumour, the only difference being a more pronounced flattening of the columnar epithelium in the most distended glandular formations of the transferred tumours. The number of mitoses is high with many atypical forms. The histological and cytological appearance of the demonstrated tumour growth of the liver and mesentery in transfer no. 3 is the same as described above.

Case 3

Primary tumour: Metastasis from the omentum majus. Adenocarcinoma with intermediate differentiation. The cells are columnar to polygonal. Moderate infiltration with granulocytes and lymphocytes.

Transfer: no take.

Case 4

Primary tumour: Adenocarcinoma with intermediate differentiation. The cells are mainly polygonal. The number of mitoses is moderate with some atypical forms. The stroma is sparce with moderate infiltration with lymphocytes and some granulocytes.

Transfer: no take.

Case 5

Primary tumour: Adenocarcinoma with intermediate differentiation. The cells are columnar showing a moderate number of mitoses. The stroma is sparce with abundant infiltration with granulocytes, many of which are eosinophil, and lymphocytes.

Transfers: Close similarity to the adenomatous structures of the primary tumour. In larger tumours extensive necrosis of the central part. Moderate number of mitoses. The stroma is more scanty with a relatively pronounced infiltration with neutrophil granulocytes and mononucleated cells.

Case 6

Primary tumour: Adenocarcinoma with intermediate differentiation. The cells are columnar with few mitoses. In some areas intra- and extracellular mucin is dominant. The stroma is rather abundant with heavy infiltration with granulocytes, many eosinophil, and lymphocytes (Fig. 4 a).

Transfer: The tumour tissue shows close similarity to the adenomatous parts of the primary tumour. Cytologically there is full correspondance with the primary tumour. The stroma is scarce with few neutrophil granulocytes and large mononucleated cells (Fig. 4 b). Necroses are extensive in the central part of the larger tumours.

Case 7

Primary tumour: Poorly differentiated adenocarcinoma. In the luminal part rather well shaped adenomatous structures with mucin production, but towards the serosal side dominated by solid tumour islands. The cells of the adenomatous formations are columnar, but in the solid areas the tumour cells are polygonal. Moderate number of mitoses with some atypical forms. Sparce stroma with only slight lymphocytic infiltration (Fig. 5 a).

Transfers: The picture is that of a solid anaplastic tumour histologically and cytologically corresponding to the most poorly differentiated parts of the primary tumour. The stromal component is minimal and there is no cellular inflammatory infiltration (Fig. 5 b).

Case 8

Primary tumour: Adenocarcinoma of intermediate differentiation. The tumour cells are columnar. High number of mitoses with a few atypical forms. The stroma is abundant. There is a poor cellular inflammatory infiltration consisting mainly of histiocytes and neutrophil granulocytes but with very few lymphocytes and plasma cells.

Transfers: The material taken for histo-

the tumours that did show take The negative outcome in these cases may be due to the technical procedure as the suspension method was used

Our increasing knowledge of the mode of tumour growth during transfers will possibly increase the percentage of takes in that tumour tissue for transfers is now taken from peripheral histologically vital parts of the tumours

It is a peculiar feature that these carcinomas which in the human host have a malignant growth pattern in the mouse mutant Nude grow as local well circumscribed apparently benign tumours without invasive growth but otherwise with the histological and cytological characteristics of the primary tumours Only in three instances disseminated tumour growth has been demonstrated in the abdominal cavity following intraperitoneal injection of tumour cell suspensions The tumour growth observed in liver and mesentery is probably a multifocal take and not metastases It is remarkable that no metastases have been observed following subcutaneous inoculation even in the most longstanding transfers The localized growth may prove an advantage when tumours are used for therapeutic studies

Another reason to prefer subcutaneous implantation of tumour blocks in the lateral abdominal wall is that this localization allows easy direct observation of tumour growth whereas tumour growth following intraperitoneal injection of tumour cell suspensions is difficult to recognize

The appearance of tumour tissues in the hosts is that of the primary tumours being very constant during transfers Only in one case (no 6) does the degree of differentiation seem to be lower in the second transfer (Figs 4 a + b) This apparent change may be a real one However two other possibilities have to be considered The most probable explanation is that the inoculum represents a poorly differentiated area not represented in the blocks taken for microscopy from the primary tumour This stresses the importance of taking out closely adjacent areas of the operative specimens for microscopical examination in future studies But secondly also a loss of stromal components in the transfers could explain the more solid epithelial appearance

The present study confirms our theory of the immune deficiencies in the mouse mutant Nude allowing the use of this laboratory animal as a host for malignant human tumours

It is important that the hosts are untreated and that they can be bred relatively easily

The fact that such transplanted tumours can be maintained and passed serially, and that the microscopical appearance seems to be constant and identical with that of the primary tumours strongly supports the concept of this model being suitable for the study of tumour immunity and for the evaluation of therapeutic agents

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We thank surgical department I for the tumour material and admission to clinical data

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logical examination (biopsy) is very scanty It consists of a few tumour islands and fibrous septa with a minimal infiltration of neutrophil granulocytes and mononucleated cells The histological and cytological picture corresponds well with the primary tumour, but the tumour material is too scanty to allow more extensive conclusions

Case 9

Primary tumour Moderately differentiated adenocarcinoma The tumour cells are uniform columnar The number of mitoses is high with a few atypical forms The stroma is sparce with slight infiltration of lymphocytes plasma cells and histiocytes

Transfers Moderately differentiated adenocarcinoma with a cytological picture corresponding to the primary tumour Scanty stroma with a few infiltrating neutrophil granulocytes and large mononucleated cells

DISCUSSION AND CONCLUSIONS

Heterotransplantation of malignant tumours to experimental animals will generally result in rejection of the tumour graft Well known exceptions from this general rule are cases where the animal host has been made immunologically unresponsive (neonatal thymectomy sublethal X ray irradiation steroid treatment immunologically tolerant animals) or to immunologically unresponsive locations (anterior eye chamber hamster cheek pouch or behind the blood liquor barrier) Successful heterotransplantation of one human malignant tumour to untreated Nude mouse mutants suffering from recessive thymic aplasia has been reported previously (5)

This paper reports growth of 6 human carcinomas of the colon and rectum in the mouse mutant Nude out of 9 consecutive transplantations Out of these tumours 5 have been transplanted serially for 2–8 transfers so far It is thus further proved that this mutant can be used for experimental work with carcinomas of the colon and rectum

Preliminary investigations with heterotransplantation of other types of human malignant tumours (malignant melanoma squamous cell carcinomas) have also been successful (6)

Greene (2) performed a retrospective study of heterotransplantation of human malignant tumours to the anterior eye chamber of normal mice This study revealed a relation between incidence of take and prognosis of the patients

The present material is too small to allow any conclusions as to the influence of donor age sex clinical course location and spread of tumour and origin of tumour graft i e primary tumour or metastases But our findings do allow some other conclusion

The degree of differentiation of the donor tumours does not seem to have any influence as growth has been obtained with both well intermediately and poorly differentiated tumours The time from removal to implantation of tumour tissue does not seem to be critical within the intervals used as take has been obtained after 45 minutes whereas tumour grafting has failed in one case after only 20 minutes This is in full accordance with the findings of *Greene* (3) who successfully transplanted human brain tumours to the brains of laboratory animals—the longest interval between operation and transfer being 90 hours Tumour growth has been obtained initially both after implantation of tumour blocks subcutaneously and after injection of tumour cell suspensions subcutaneously and intraperitoneally The results seem to be slightly better with solid tumour blocks so that this method has been used in later transfers With this method the percentage of take is reasonable so as to allow this model to be used in experimental tumour studies

One tumour (case no 8) failed to grow in transfer no 2 probably due to too small inocula Case no 5 did not show take in the third transfer probably due to extensive necrosis of the inocula as verified on subsequent histological examination (*Fig 6*)

The three tumours which did not grow did not differ in histological appearance from

The titre of cytotoxic antibodies was determined by the microtechnique of *Kissmeyer Nielsen & Kjerbye* (1967) Lymphocytes were harvested from heparinized blood according to the technique described by *Boyum* (1968) Details concerning these procedures are reported in another paper (Ahrons & Lund)

Kidney transplantation was performed by end to-side anastomosis between the renal vessels of the left donor kidney and the abdominal aorta and the inferior vena cava of the recipient (*Lund* 1970) Immediately after removal, the donor kidney was perfused with 10 ml of cold saline in order to reduce the period of warm ischaemia Warm ischaemia lasted for 1–2 minutes the period of cold ischaemia averaged 40 minutes (range 30 50 minutes) In 5 cases heparin (500 *i u*) was added to the perfusion fluid in 8 cases papaverine (8 mg) and in 6 heparin plus papaverine was added Saline alone was used in 38 cases

After re-establishment of the circulation, the graft was observed for 1–6 hours before uretero vesical anastomosis was carried out One of the recipients own kidneys was not handled in order to avoid uraemia Thirteen of these untouched kidneys were removed simultaneously with the allografts and served as controls The other one was used as donor kidney in another transplantation No immunosuppressive therapy was given Second set kidney transplantation was performed in 14 cases In 12 cases the second set allograft was derived from the same donor as the first one whereas in 2 cases the second set graft was derived from another rabbit The serum of these 2 recipients showed demonstrable cytotoxic antibodies against donor leucocytes The second-set kidney was anastomosed to the abdominal aorta and the inferior vena cava at a site 1 cm higher up than that of the first set Only allografts with apparently well functioning vascular anastomosis were included in this part of the study

The kidney grafts were removed for microscopic examination at intervals of from 1 hour to 5 days after operation Tissue specimens were fixed in 4 per cent buffered formaldehyde and sections of paraffin embedded material were stained with haematoxylin-eosin, phosphotungstic acid haematoxylin, Lendrum's stain for fibrin, periodic acid Schiff, picrosirius red and Unna Pappenheim stain (methyl green pyronine) Immediately after preparation the specimens were examined by one of us (BL) Later on they were examined in a pool with specimens from other experiments by OMJ, who had not seen any of the preparations before and who was not aware of the experimental data at the time of examination The morphological findings were semiquantitated (Table 2)

TABLE 1 *Number of Skin Grafts before Renal Transplantation*

Number of skin grafts	Number of recipients
2	2
3	4
4	12
5	8
6	13
9	18

RESULTS

The results of the serological investigations and the blind analysis of morphological alterations in 57 allografts from pre-sensitized recipients, removed 1–5 days after transplantation, are shown in Table 2 The titre of cytotoxic antibodies recorded in Table 2 was determined 1 to 3 days before renal transplantation None of the rabbits had circulating cytotoxic antibodies against donor leucocytes before skin grafting

Eleven allografts removed after an observation period ranging from 1 to 6 hours after re-establishment of circulation were initially well functioning but soon lost tension, became brownish-cyanotic and stopped production of urine Three of these developed either circulatory collapse or respiratory insufficiency, whereas the general state of the other 8 animals was satisfying No glomerular or vascular changes were found in these grafts Four of the grafts showed hyperaemia of the juxtamedullary zone of the cortex and one demonstrated graft oedema Thrombosis of the intrarenal vessels was not seen

Cellular Infiltration

Mononuclear cell infiltrates in close association with the veins near the corticomedullary junction and with the peritubular capillary network were found in 18 allografts During the first 2 days after transplantation the cell infiltrates consisted mainly of small lymphocytes From about the third day, larger lymphocytes with a lighter nucleus and more abundant cytoplasm domi-

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RENAL TRANSPLANTATION IN RABBITS

IV Morphological Alterations in Allografts from Recipients Presensitized by Multiple Skin Grafts

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The histological changes in 57 renal allografts from rabbits presensitized by multiple skin grafts have been studied semiquantitatively No histological changes, except oedema, were observed in 11 allografts removed within 6 hours after transplantation because of clinical rejection Eleven allografts removed 1-5 days after transplantation showed the pattern of hyperacute rejection extensive glomerular microthrombosis and cortical necrosis Circulating cytotoxic antibodies against donor leucocytes did not always induce hyperacute rejection but they were found in all the cases of hyperacute rejection tested for their presence The most conspicous difference between the allograft reaction in sensitized and in non sensitized recipients was the early appearance and the increased frequency of exudative glomerulitis and glomerular microthrombosis In addition proliferation of the endothelial cells of the glomeruli and vessels and vasculitis were more frequent in sensitized rabbits The results indicate that presensitization accelerates allograft rejection in rabbits and suggest that this process, which in several cases was morphologically similar to human hyperacute allograft reaction is to some extent related to circulating antibodies

Previously grafted recipients usually exhibit accelerated allograft rejection In man, hyperacute, immediate rejection seems to be related to the presence of circulating antibodies to donor leucocytes or thrombocytes The present study in rabbits was undertaken in order to elucidate the effect of presensitization by skin grafts on the renal allograft reaction It has previously been shown that skin transplantation in rabbits produces cytotoxic antibodies against donor leucocytes (*Lund & Ahrons* 1970) The morphological alterations in the renal allografts from rabbits treated with multiple skin grafts were compared with those in allografts from non-treated rabbits (*Lund & Myhre Jensen* 1970)

Received 12 viii 70

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MATERIAL AND METHODS

Randomly bred rabbits weighing 2 3 kg were used Donor and recipient were chosen from out bred strains of New Zealand white rabbits and Brown Lop eared rabbits Rabbits were fed a common laboratory diet with additional fresh vegetables and allowed drinking water ad libitum

Prior to kidney transplantation the recipient was sensitized by multiple skin grafts from the prospective donor Full thickness circular skin grafts measuring 1 1 cm in diameter were taken from the back of the donor s ear and applied to the back of the recipient s ear In the start of the study skin grafts were sutured by continuous 7-0 atraumatic silk Later only two sutures were applied and the grafts pressed firmly against the graft bed for three minutes and then fixed by Nobecutan® spray

Each recipient received from two to nine skin grafts from the prospective donor (Table I The period of sensitization lasted for from 6 weeks to six months The interval between each skin grafting varied from 1 to 6 weeks

	246	1					—	[illegible]	[illegible]	[illegible]
	247	17	+ +	+ + +	+ +	+ + +	+	+ + +	+ + +	[illegible]
	248	8	+ +	+ +	—	+ +	—	—	—	—
	251	1	+	+ +	—	—	—	—	—	—
	253	1	—		—	—	—	—	+ + +	+ + +
	254	2	+ +	+	—	—	—	—	—	—
	210	1	+ +	+ + +	+ +	+ +	+ +	—	—	—
	218		+	+		—	—	—	—	—
	219		—	—	—	—	—	+ + +	+ + +	+ + + +
	231	8	+	—	—	+ + +	—	+ + +	+ + +	+ + +
	232	1	+ + +	+ +	+	+	+	—	—	—
	237	8	+	+ + +	+	+ + +	+	+	—	+
3	238	1	+ +	+ + +	—	+ +	—	—	—	—
	243	4	+ +	+ + +	—	+ + +	—	+ +	+ + +•	+ + +
	244	2	+ +	+ +	—	+	—	+	—	—
	249	4	+	—	+ +	—	—	+ + +	+ + +	+ + +
	250	—	+ +	+ +	+ +	+	+	—	—	—
	252	—	+	—	—	—	—	—	—	—
4	236	8	—	—	—	—	—	+ +	+ + +	+ + +
	213	4	—	—	—	—	—	—	+ + +	+ + + +
	225	2	—	—	—	—	—	+ +	+ +	+ + +
5	226	2	—	—	—	—	—	—	+ + +	+ + +
	242	4		—	—	—	—	—	+ + +	+ + +

(Table 2) *Key to Semiquantitative Evaluation*

	Mononuclear cell infiltr	Glomerular changes in	Vascular changes in	Necrosis
+	small infiltrates	less than 10% of the glom	a few vessels	patchy cortical necrosis
+ +	many or large perivascular	10–50% of the glomeruli	many vessels	subcapsular zone
+ + +	diffuse infiltr	more than 50% of the glomeruli	almost all vessels	partial necrosis of cortex and medulla
+ + + +				total necrosis

TABLE 2 *Morphological Alterations in 57 Renal Allografts from Rabbits Presensitized by Multiple Skin Grafts (Key to the Semiquantitative Evaluation is Given below the Table)*

Day	Rabbit	Titre	Cell infiltr	Prolif changes		Exudat changes		Thrombosis		Cortic necr
				glom	vessels	glom	vessels	glom	vessels	
	204	32	—	—	—	—	—	—	—	—
	208	2	—	—	—	—	—	—	—	—
	215	16	—	+ +	—	—	—	—	—	—
	223	8	—	—	—	—	—	—	—	—
	230s	1	—	—	—	—	—	—	—	—
2–6 hours	235	8	—	—	—	—	—	—	—	—
	235s		—	—	—	—	—	—	—	—
	239	—	—	—	—	—	—	—	—	—
	240s		—	—	—	—	—	—	—	—
	243s	4	—	—	—	—	—	—	—	—
	245	8	—	—	—	—	—	—	—	—
	209		—	—	—	—	—	—	—	—
	214	16	—	—	—	—	—	—	—	—
	223s		—	—	—	—	—	—	—	—
	224	1	—	—	—	—	—	+ +	+ + +	+ + +
	231s	32	—	—	—	—	—	—	—	+ + +
	232s	2	—	+ +	+ +	+	—	+	+ +	+ +
1	237s		—	+ + +	—	+ +	—	+ +	+ +	+ + +
	239s		—	—	—	—	—	—	—	—
	240	—	+	+	+	—	—	—	—	—
	241s		—	—	—	—	—	—	+ + +	+ + +
	247s		—	—	—	—	—	—	—	—
	249s		—	—	—	—	—	+ +	+	—
	251s		—	+ + +	—	+ +	—	—	—	—
	253s		—	—	—	+ +	—	—	+ + +	+ + +
	211	8	—	—	—	—	—	—	—	—
	212	1	—	—	—	—	—	—	—	—
	216	—	—	—	—	—	—	+	+ + +	+
	217	16	—	—	—					
	221									

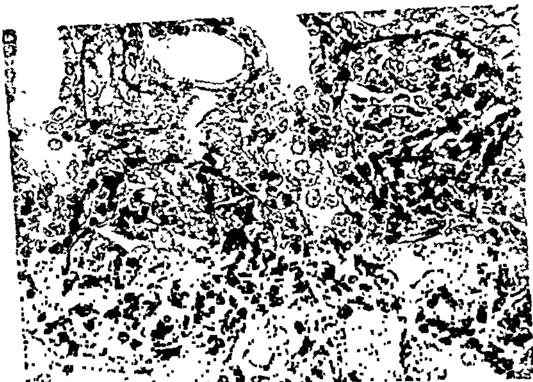


Fig 2 Allograft no 210 removed 3 days after transplantation Severe proliferative and exudative glomerulitis PAS × 400

and frequently affected 50 per cent or more of the glomeruli

Vasculitis, characterized by an inflammatory exudate consisting of polymorphonuclear leucocytes and/or necrosis of vascular walls, was seen in only 5 allografts Vasculitis was accompanied by exudative glomerulitis, proliferative glomerulitis and endarteritis in all cases (Fig 4)

Thrombosis

Glomerular microthrombosis was seen in 17 allografts accompanied by proliferative and exudative glomerulitis in 7 cases and by exudative glomerulitis alone in 3 cases Glomerular microthrombosis was accompanied by thrombosis of other intrarenal vessels in all cases but one Microthrombi were present in less than 10 per cent of the glomeruli in 5 cases and from 10 per cent to over 50 per cent in 12 cases When several glomeruli were involved, the greater part of the capillary network was usually filled with fibrinous material (Fig 5–6). When only a few glomeruli were affected, the microthrombi appeared as one or two capillary fibrin plugs (Fig 7–8)

Thrombosis of intrarenal blood vessels other than those in glomeruli was found in 22 allografts In 6 cases microthrombosis was also present Nine of the 22 allografts had cellular infiltrates and glomerular or vascular changes In 7 cases, however, no proliferative or exudative changes were demonstrated

Necrosis

Patchy cortical necrosis localized around one or a few interlobular arteries was found in 2 allografts, in both accompanied by glomerular microthrombosis

Subcapsular necrosis affecting the peripheral one or two-thirds of the cortex was

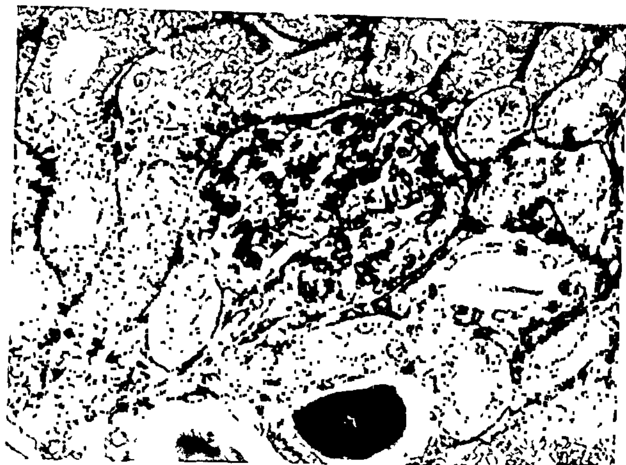


Fig 1 Allograft no 248 removed 2 days after transplantation Moderate proliferative and exudative glomerulitis PAS × 400

nated Pyroninophilic cells appeared one day after transplantation and were seen in 8 of the 18 allografts with cellular infiltrates Eosinophils were frequently found in groups of 2 or 3 cells, especially during the first 2 days Neutrophils were seen in the demarcation zone around necrotic areas as well as in inflammatory exudates in glomeruli and vessels (see below)

Proliferative Changes in Glomeruli and Vessels

Proliferative glomerulitis, characterized by swelling or proliferation of glomerular endothelial or mesangial cells (Fig 1–2), was found in 19 allografts The number of allografts with proliferative glomerulitis as well as the number of glomeruli involved in each graft increased steadily from the first day after transplantation During the first 2 days, 10–50 per cent of the glomeruli in each graft were usually affected whereas more than 50 per cent were often involved in the affected grafts on the third day Cell infiltrates were demonstrated in 14 out of the 19 cases

Endarteritis, characterized by swelling or proliferation of the vascular endothelium was demonstrated in 8 allografts (Fig 3) Endarteritis was accompanied by proliferative glomerulitis in all cases but one Endothelial proliferation was more pronounced in arteries than in veins

Exudative Changes

Exudative glomerulitis, characterized by exudation of polymorphonuclear leucocytes into the glomerular tuft or capsular space of the glomeruli, was found in 16 allografts Exudative glomerulitis was accompanied by proliferative glomerulitis in all cases but three

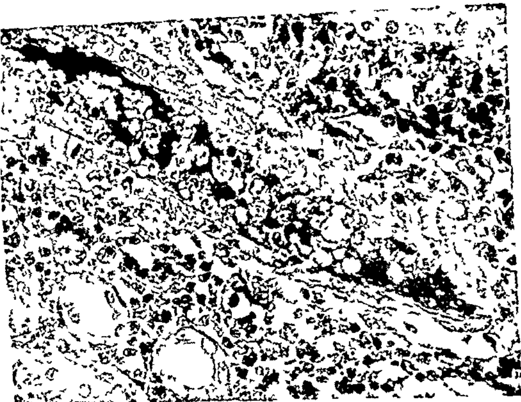


Fig 4 Allograft no 210 (as in Fig 2) Endothelial proliferation and exudation of inflammatory cells in the wall of an interlobular artery PAS × 400

DISCUSSION

In 1953 *Simonsen et al* demonstrated in the dog that a single skin graft can sensitizise a recipient to the extent that a kidney graft from the same donor is rejected more violently than usual Recently *Loewenhaupt* & *Nathan* (1969) showed that sensitization of dogs by multiple skin grafts could provoke e rapid rejection of kidney grafts Histo ogically these grafts presented a pattern haracterized by cortical necrosis and glom rular microthrombosis similar to the hyper cute allograft reaction in human kidney ransplants described by *Kissmeyer Nielsen t al* (1966) *Mosely et al* (1966) con cluded that the simultaneous presence of skin and kidney grafts from the same donor resulted in prolonged survival of the skin but a marked decrease in the survival of the kidney graft *MacDonald et al* (1970) also found that dogs presensitized by multiple skin grafts displayed rapid rejection of kidney grafts and increased resistance to perfusion during the first 24 hours after revascularization neutrophilic infiltration of the cortex, and thrombosis of the smaller cortical ar teries

Sensitization by skin grafts however, does not always result in accelerated rejection of kidney grafts *Feldman* & *Lee* (1969) con cluded from their experiments with renal transplantation in rats presensitized with skin grafts that any clinical or histological signs of acceleration of the rejection process were not demonstrable *White* & *Hildeman* (1969) found that kidney allografts in immunologic ally non suppressed rats generally persisted for extended periods of time in spite of se vere acute rejection of multiple skin allografts from the same donor strain The frequency of accelerated rejections in the experiments

found in 2 cases, in both associated with proliferative and exudative changes in glomeruli and vessels

Partial necrosis of the kidney with involvement of cortex and outer medullary zone was found in 16 allografts. In 10 of these, no proliferative change or lymphocytic infiltration was seen

Total necrosis of the kidney was seen in 2 allografts In one of these, glomerular microthrombosis was present in about 50 per cent of the glomeruli.

The necrotic areas of the grafts were demarcated by a zone of polymorphonuclear leucocytes

Varying degrees of tubular degeneration could be demonstrated in most of the allografts Oedema was seen in 38 and severe hyperaemia in 7 allografts

No glomerular microthrombosis was discovered in allografts perfused with heparin and papaverine (209, 210, 211, 212, 213 and 214). In the groups treated with heparin (5 cases) or papaverine alone (8 cases), glomerular microthrombosis occurred in one case in each group

Second-set transplants Three out of the 14 second-set allografts showed glomerular microthrombosis In 2 of these animals, microthrombosis had also been demonstrated in the first-set transplant Four rabbits (231, 241, 243 and 247) had glomerular microthrombosis in the first-set allograft, but not in the second-set. One rabbit (232) had glomerular microthrombosis in the second set, but not in the first-set transplant

Controls No glomerular or vascular changes and no cellular infiltration were discovered in any of the 13 pre-sensitized recipients' own kidneys removed simultaneously with the kidney graft

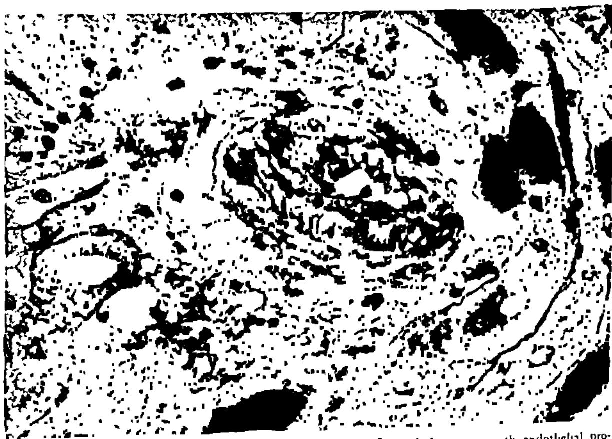


Fig 3 Allograft no 232s removed 1 day after transplantation Interlobular artery with endothelial proliferation PAS × 400

rculating cytotoxic antibodies against the ells of the donor

Allografts removed during the first 6 hours fter revascularization because of clinical rejection showed no morphological alterations ndicative of hyperacute rejection Perhaps ejection was caused by the release of vasoonstrictive agents in the damaged kidney or ecause the renal vasculature had become ypersensitive to circulating vasoconstrictive ubstances (*Busch et al* 1969, *Hollenberg et al* 1968 *MacDonald et al* 1970) The arly changes in kidney grafts in sensitized nimals have been studied by electron microscopy by *Loewenhaupt & Nathan* 1969 in dogs Platelet aggregation on the vascular endothelium was observed 10 minutes after transplantation In the 5 hour specimens, platelet thrombosis was evident and some of the platelet pseudopods protruded through the endothelium and touched the basement membrane The degranulation of the plate lets with free cytoplasmatic granules in the vascular lumen rises the possibility that vasoactive substances known to be present in platelets may contribute to vasoconstriction *Busch et al* (1969) suggested that vasoactive polypeptides produced by fibrinolytic activity may be an alternative factor leading to vasospasm In contrast to *Loewenhaupt & Nathan*'s and to our observations, *MacDonald et al* 1970 saw focal aggregation of erythrocytes in glomerular and peritubular capillaries 5 minutes after revascularization Thirty minutes after transplantation neutrophils were present in increased number in the capillaries and fibrin platelet thrombi were seen in small cortical arteries

The significance of sensitization by skin grafts prior to kidney transplantation is illustrated in Table 3 It appears that the frequency of glomerular and vascular changes is increased in the sensitized rabbits The increased frequency of glomerular microthrombosis and exudative glomerulitis in presensitized recipients is the most impressive dif



Fig 6 Details of Fig 5 Glomerular capillaries with fibrinous microthrombosis PTAH × 400

12*

of *Loewenhaupt & Nathan* and *MacDonald et al* is not stated

Another uncertain point in the animal experiments is the influence of an accidental major incompatibility between donor and recipient, acting together with skin graft sensitization In the case of kidney transplantation in man rapid rejection of kidney grafts with a pattern similar to hyperacute rejection has been reported to occur in cases of ABO incompatibility (*Porter* 1967) In the rabbit, *Cohen et al* (1962) have demonstrated that blood group incompatibility does not influence second set survival of skin grafts Blood groups have not been examined in our study The frequency of accidental major incompatibility, however, should be expected to be about the same as that seen in *118 kidney transplantations in non sensitized* rabbits, (*Lund & Myhre Jensen* 1970)

The titre of cytotoxic antibodies in the serum of the recipient, directed against leucocytes in the specific donor was used as a measure of the state of sensitization because this parameter has been shown to be important in human kidney graft recipients in whom a possible presensitization may be due to multiple blood transfusion or pregnancies (*Kissmeyer Nielsen et al* 1966) Cytotoxic antibodies were generally demonstrable after 4 skin grafts, more than 4 skin transplants resulted only occasionally in an increase in titre (*Lund & Ahrons*)

It appears from Table 2 that the presence of cytotoxic antibodies did not regularly cause accelerated or hyperacute rejection of kidney grafts But on the other hand, all such allografts as displayed a histological pattern characteristic of hyperacute rejection did have circulating cytotoxic antibodies This *finding corresponds to the findings of Morris et al* (1968) concerning human allografts They reported that hyperacute rejection did not always occur in spite of the existence of

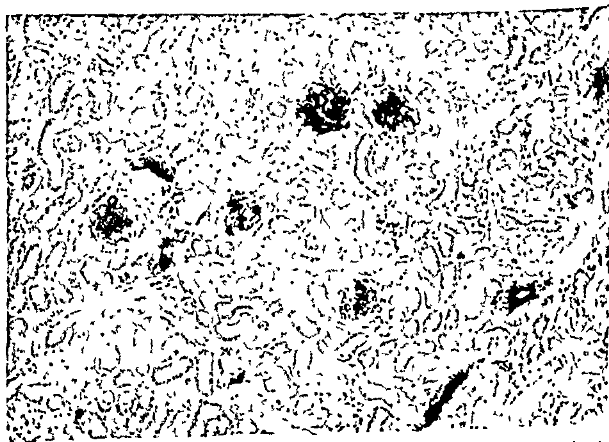


Fig 5 Allograft no 222 removed 2 days after transplantation Glomerular microthrombosis and cortical necrosis PTAH × 100

other side The finding of fibrin split products in urine during the first 14 days after transplantation in most kidney recipients and the reappearance of the split products in relation to rejection episodes suggest that fibrin deposition and fibrinolysis are involved in the allograft reaction (*Colmann et al* 1969 *Busch et al* 1969) Consequently individual variations in the fibrinolytic activity of kidney tissue might be one of the factors responsible for the variations in the histological pattern observed in kidney allografts The fibrinolytic activity of kidney allografts in rabbits is presently under study

In conclusion a semiquantitative study of the allograft reaction in rabbits has shown that sensitization provokes a high frequency of glomerular microthrombosis and cortical necrosis and an acceleration of the proliferative and exudative changes in glomeruli and blood vessels In all cases with glomerular microthrombosis and cortical necrosis circulating antibodies were found Circulating leucocytotoxic antibodies, however, do not always result in rapid rejection in the rabbit

The histological pattern in kidney allografts from non sensitized rabbits is generally characterized by more pronounced proliferative than exudative changes in glomeruli and blood vessels and the relatively late appearance of large lymphocytes in the perivascular infiltrates (*Lund & Myhre Jensen* 1970) Morphology in sensitized rabbits is characterized by the simultaneous appearance and similar extent of proliferative and exudative changes the early appearance of large lymphocytes in perivascular infiltrates and an increased frequency of glomerular microthrombosis

The study was aided by grants from the *"Danish State Research Foundation"* and *F L. Smidth & Co Foundation*

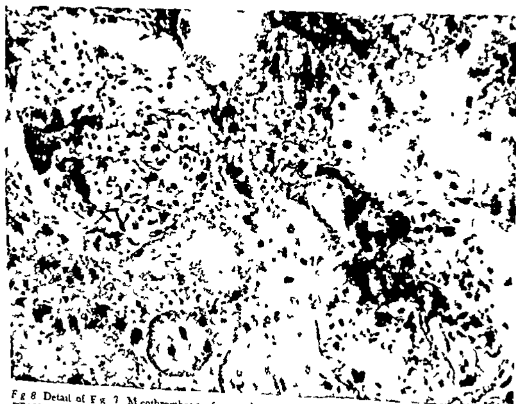


Fig 8 Detail of Fig 7 Microthrombosis of two glomeruli affecting the vascular pole in one glomerulus PTAH × 400

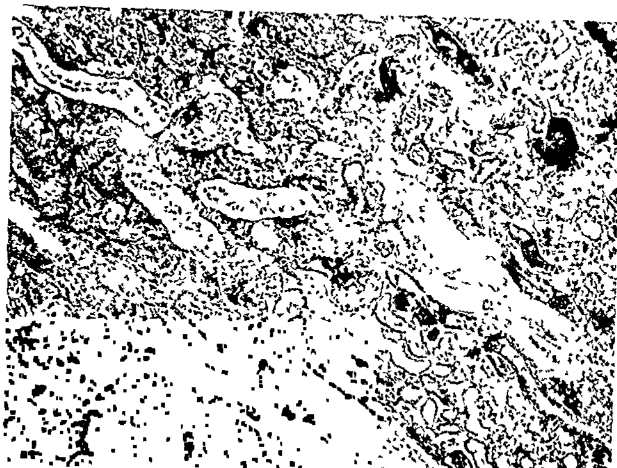


Fig 7 Allograft no 247 removed 2 days after transplantation Vascular and glomerular microthrombosis and cortical necrosis PTAH × 100

ference The frequency of lymphocytic infiltration is about the same in the two groups The lymphocytes in the kidney grafts from non-sensitized recipients are, however, mainly small lymphocytes, in the sensitized recipients, large lymphocytes were found more often Large lymphocytes dominated the cell infiltrates in allografts from non sensitized recipients from about the fifth day after transplantation Based on this comparison we may conclude that sensitization by multiple skin grafts frequently accentuates and accelerates the allograft reaction (*Lund & Myhre Jensen* 1970)

Thrombosis and microthrombosis in kidney grafts represent a break in the homoeostasis of clotting mechanisms involving fibrin deposition on one side and fibrin clearing on the

TABLE 3 *The Frequency (in Percentage) of Morphological Alterations in Renal Allografts Removed 1–3 Days after Transplantation from 41 Sensitized and 36 Non Sensitized Rabbits*

	Total number	Cellular infiltr	Prolif changes glom	Prolif changes vessels	Exudat changes glom	Exudat changes vessels	Thrombosis glom	Thrombosis vessels	Necrosis	N char
Sensitized rabbits	41	43	43	19	38	12	36	41	43	2
Non sensitized rabbits	36	48	33	8	8	3	3	20	20	3

Acta path microbiol scand Section A 79, 183-192 1971

RENAL TRANSPLANTATION IN RABBITS

V *Morphological Alterations in Allografts from Recipients Presensitized by Donor Kidney Homogenate*

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The histological changes in 46 specimens from 18 renal allografts performed in rabbits presensitized by 5-6 injections of donor kidney homogenate have been analysed semi quantitatively Cytotoxic antibodies against donor leucocytes could be demonstrated in all presensitized rabbits at the time of transplantation Two of the allografts showed a morphological pattern characteristic of hyperacute rejection i e extensive glomerular microthrombosis and cortical necrosis Compared with allografts from non sensitized recipients, the frequency of proliferative glomerulitis, thrombosis including glomerular microthrombosis, and necrosis was increased, the frequency of lymphocytic infiltration was about the same The frequency of morphological alterations was lower in homogenate treated recipients than in allografts from rabbits presensitized by multiple skin grafts from the donor

The histological alterations in renal autografts, allografts, and in allografts from rabbits presensitized by multiple skin grafts have previously been described Sensitization of recipients by skin grafts frequently accelerated the proliferative and exudative changes in glomeruli and blood vessels and increased the frequency of glomerular microthrombosis and of thrombosis of other intrarenal blood vessels

The effect of sensitization by multiple skin grafts varied, however, from rabbit to rabbit In order to further study the characteristics of sensitization, the morphological alterations in renal allografts from recipients presensitized by multiple injections of crude kidney homogenate from the donor have been analysed and compared with those in allografts from non sensitized and skin sensitized recipients (*Lund & Myhre Jensen* 1970 & 1971, III, IV)

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MATERIAL AND METHODS

Adult rabbits of both sexes, weighing 2-3 kg, were used The donor and recipient were chosen from different strains of outbred New Zealand white rabbits and brown Lop eared rabbits The rabbits were fed a common laboratory diet supplemented with fresh vegetables and allowed drinking water ad libitum

Sensitization

Prior to kidney transplantation the recipient was sensitized by kidney homogenate from the prospective donor The right kidney of the donor was cut into small pieces after removal of the capsule and homogenized in a Warring blender for 5 minutes Two ml of Freund s complete adjuvant and 1 ml of normal saline were added to two ml of the crude homogenate and mixed for another 3 minutes Three ml of this mixture was injected sub-

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							–		+ + +	+ + +
3	283	8		+		–	–	–	–	–
	285	8				–		+ + +	+ + +	+ + +
	285s	8	+	+		–	–	–	–	–
	287	4	+ +	+		–	–	–	–	–
	291	4	+ +	+		–	–	–	+	+
	271	8	–	–	–	–	–	–	–	–
	277	8	+ +	+ + +		–	–	+	+	–
4	283	8	+	+		–	–	–	+	+
	285	8	–	–		–	–	+ + +	+ + +	+ + + +
	291	4	+ +	+ +	–	–	+	–	+ +	+
6	277	8	+ + +	+ + +	–	–	–	+	+ +	–

(Table 1) *Key to Semiquantitative Evaluation*

	Mononuclear cell infiltr	Glomerular changes in	Vascular changes in	Necrosis
+	small infiltrates	less than 10 % of the glom	a few vessels	patchy cortical necrosis
+ +	many or large perivasculr	10–50 % of the glomeruli	many vessels	Subcapsular zone
+ + +	diffuse infiltr	more than 50 % of the glomeruli	almost all vessels	partial necrosis of cortex and medulla
+ + + +				total necrosis

TABLE 1 *Morphological Alterations in 46 Specimens from 18 Renal Allografts in Rabbits Pre Sensitized by Donor Kidney Homogenate (See below for Key to Semiquantitative Evaluation)*

Day	Rabbit	Titre	Cell infiltr	Prolif changes glom	Prolif changes vessels	Exudat changes glom	Exudat changes vessels	Thrombosis glom	Thrombosis vessels	Necrosis
2–6 hours	265	8	—	—	—	—	—	—	—	—
	283	8	—	—	—	—	—	—	—	—
	285	8	—	—	—	—	—	—	—	—
	289	4	—	—	—	—	—	—	—	—
	291	4	—	—	—	—	—	—	—	—
1	255	8	—	—	—	—	—	—	—	—
	259	4	—	+ + +	—	—	—	—	—	—
	261	1	—	—	—	—	—	—	—	+ + +
	263	1	—	—	—	—	—	—	—	—
	269	4	—	—	—	—	—	—	—	—
	271	8	—	—	—	—	—	—	—	—
	277	8	+	+ +	—	—	—	—	—	—
	279	4	—	—	—	—	—	—	—	—
	281	4	+	—	—	—	—	—	—	—
	283	8	—	—	—	—	—	—	—	—
	285	8	—	—	—	—	—	+ +	+ + +	—
	285s	8		—	—	—	—	—	—	—
	287	4	+ +	+	—	—	—	—	—	—
	289	4	—	—	—	—	—	—	—	—
	291	4	+	+	—	+	—	—	—	—
	291s	4	—	—	—	—	—	—	—	—
2	255	8	—	—	—	—	—	—	—	—
	259	4		+ +	—	—	—	—	—	+
	261	1	—	—	—	—	—	—	+ +	+ +
	263	1	+ +	+ +		—	—	—	—	—
	269	4		—	—	—	—	—	—	—
	273	8		—	—	—	—	+ + +	+ + +	+ + +
	283	8		—	—	—	—	—	—	—

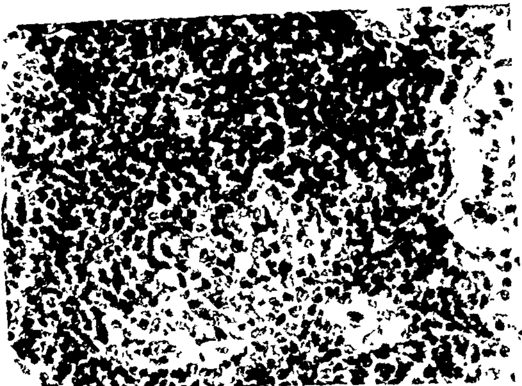


Fig 1 Biopsy from allograft no 277 removed 4 days after transplantation Heavy infiltration by lymphocytes around interlobular artery HE × 400

nuclear leucocytes and fibrin into the glomerular tuft or Bowmann's space was only found in two specimens and in both was present in less than 10 per cent of the glomeruli In one of the cases (no 291), 3 additional biopsies did not show any sign of exudative glomerulitis

Vasculitis characterized by inflammation and/or necrosis of the vascular wall was seen in only 1 specimen (no 291) and in only a few vessels

Thrombosis Glomerular microthrombosis was seen in 3 allografts In one of these microthrombosis was present in less than 10 per cent of the glomeruli proliferative glomerulitis and perivascular lymphocytic infiltration were also found In the 2 other cases, microthrombosis was found in more than 50 per cent of the glomeruli, both grafts showed cortical necrosis which included the outer medullary zone (Figs 4–5)

Thrombosis of the intrarenal blood vessels was found in 7 cases Proliferative glomerulitis and perivascular lymphocytic infiltration were observed in 3 of these Glomerular microthrombosis accompanied the vascular thrombosis in an additional 2 cases

Necrosis Patchy, cortical necrosis affecting the tissue around one or a few interlobular arteries was observed in 2 allografts, in both accompanied by proliferative glomerulitis and lymphocytic infiltration

Partial necrosis of the kidney with involvement of most of the cortex and outer medullary zone was seen in 5 allografts One of these (no 285) later developed total necrosis Tubular degeneration was found to a varying extent in most of the allografts Oedema was present in 10, severe hyperaemia in 2 allografts

Controls Fourteen of the recipients' own kidneys were included in the blind test evaluation Proliferative glomerulitis was demonstrated in one of the kidneys, whereas 5 of

cutaneously into the medial aspect of the recipients femur

The rest of the crude homogenate (without adjuvant) was divided into 5 portions and frozen at —70° C From 1 to 2 weeks after the first injection 2 ml of a mixture containing 2 ml frozen homogenate 1 ml of Freund's complete adjuvant and 2 ml saline was given subcutaneously During the following 3 weeks the recipients were given weekly injections of 1 ml crude homogenate in 1 ml saline but without Freund's adjuvant Seven recipients received a booster dose of 2 ml homogenate and 1 ml saline intraperitoneally one month after the fifth subcutaneous injection Twenty two recipients were sensitized 6 of the recipients and 2 donors however died of Pasteurella multicoides pneumonia during the period of sensitization

Serological investigation The titre of cytotoxic antibodies directed against donor leucocytes was examined 1 to 10 weeks after the primary injection of homogenate The last determination was performed 1 3 days before kidney transplantation The details of these procedures are reported in another paper (*Lund & Ahrons*)

Kidney transplantation was performed via an end to side anastomosis between the renal vessels of the left donor kidney and the abdominal aorta and inferior vena cava of the recipient (Lund 1970) Immediately after removal the donor kidney was perfused with 10 ml of cold saline to reduce the period of warm ischaemia Warm ischaemia lasted for 1 2 minutes cold ischaemia averaged 40 minutes (range 30 60 minutes) After re establishment of the circulation the graft was observed for 1–4 hours for colour changes and changes in urinary output before the ureterovesical anastomosis was carried out The recipients own kidneys remained untouched until they were removed simultaneously with the allografts and used as controls No immunosuppressive therapy was given Second set kidney transplantation from another donor was carried out in 2 cases Prior to second set transplantation the 2 recipients were examined for cytotoxic antibodies against a panel of 5 rabbits and the rabbit with the highest titre was used as the donor in the second transplantation The second graft was anastomosed to the abdominal aorta and inferior vena cava at a site 1 cm higher up than the first graft 1 2 weeks after removal of the first graft

Morphological alterations in the renal allografts were studied by serial biopsies and by removal of allografts at intervals of from 3 hours to 6 days after transplantation Cortical wedge biopsies were obtained at intervals of from 1 hour to 3 days after transplantation under general anaesthesia (Nembutal®/N_2O/O_2) alternately from the upper and lower parts of the kidney at intervals of 1 day

Specimens of tissue for microscopy were fixed in 4 per cent buffered formaldehyde paraffin sections were stained with haematoxylin-eosin phosphotungstic acid haematoxylin Lendrum's stain for fibrin periodic acid Schiff picrosirius red and methyl green pyronine (Unna Pappenheim)

The specimens were examined immediately after preparation by one of us (B L) Later they were examined in a pool comprising specimens from kidney autografts and allografts by OMJ who had not seen any of the preparations before and who was not cognizant of the experimental data at the time of examination The evaluation was made semiquantitatively

RESULTS

The results of serological examination and blind test analysis of 46 specimens from 16 primary and 2 second set grafts are shown in Table 1 Cytotoxic antibodies were demonstrated in the serum of all rabbits prior to kidney transplantation Only allografts with apparently well functioning vascular anastomosis are included

Cellular infiltrations Infiltration by mononuclear cells was found in 9 out of the 18 allografts The infiltrates were localized around vessels in the corticomedullary zone and the serial biopsies showed that the infiltrates during the first days were mainly composed of small lymphocytes with dense nucleus and sparse cytoplasm (Fig 1) These were later replaced by larger lymphocytes with paler nucleus and more abundant cytoplasm

Pyroninophilic cells were found in 7 allografts and eosinophils in most Polymorphonuclear leucocytes were seen in the demarcation zone of cortical necrosis

Proliferative changes in glomeruli and vessels Proliferative glomerulitis characterized by swelling or proliferation of endothelial and mesangial cells was found in 9 allografts (Figs 2 and 3) In four of these cases however, less than 10 per cent of the glomeruli in each section were affected

Endarteritis characterized by swelling or proliferation of the vascular endothelium was not found in any of the allografts

Exudative changes Exudative glomerulitis characterized by exudation of polymorpho-

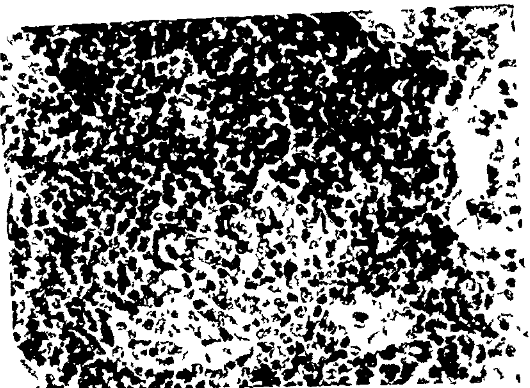


Fig 1 Biopsy from allograft no 277 removed 4 days after transplantation Heavy infiltration by lymphocytes around interlobular artery HE × 400

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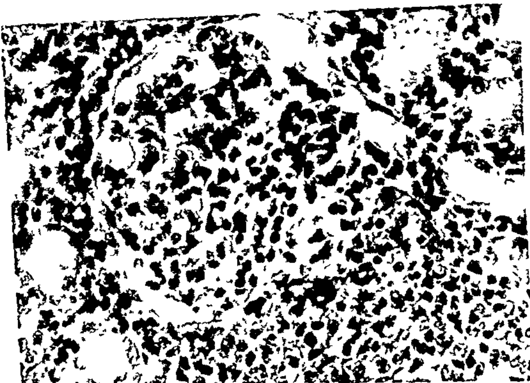


Fig 3 Allograft no 277 (as in Fig 1 2) removed 6 days after transplantation. Pronounced proliferative glomerulitis PAS × 400

cortical necrosis are frequent A pattern characterized by widespread glomerular microthrombosis and cortical necrosis similar to the histological pattern seen in cases of hyperacute rejection of human renal allografts was observed in 4 out of 118 allografts

In Table 2 the frequency of histological changes in allografts removed 1–3 days after transplantation from recipients sensitized by multiple injections of kidney homogenate is compared with the frequency of lesions in grafts from non sensitized rabbits removed within the same time interval after transplantation When several biopsies from the same graft were available the most recent one was used As appears from the table, the frequency of proliferative glomerulitis, thrombosis—including glomerular microthrombosis—and necrosis is increased in the presensitized rabbits The frequency of lymphocytic infiltration endarteritis exudative glomerulitis and vasculitis is about the same or less than that in non sensitized rabbits

TABLE 3 *The Frequency (in Percent) of Morphological Alterations in Renal Allografts Removed 1 3 Days after Transplantation from Recipients Pre-Sensitized by Donor Kidney Homogenate and by Multiple Skin Grafts*

	Total	Cell infiltr	Prolif changes glom	Prolif changes vessels	Exudat. changes glom	Exudat. changes vessels	Thrombosis glom.	Thrombosis vessels	Necrosis	N[illegible] cha[illegible]
Homogenate	17	47	53	0	12	6	12	30	35	[illegible]
Skin	41	43	43	19	38	12	36	41	43	[illegible]

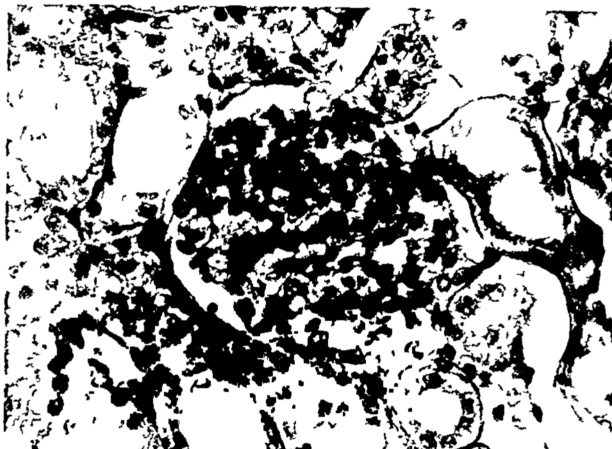


Fig 2 Biopsy from allograft no 277 (as in Fig 1) removed 1 day after transplantation Moderate proliferative glomerulitis PAS × 400

the control kidneys presented small areas of lymphocytic infiltration No other abnormalities were discovered in these non transplanted kidneys

DISCUSSION

A study of the effect of presensitization with donor kidney homogenate on the allograft reaction should be based on an analysis of the histological changes in allografts from non sensitized recipients (*Lund* & *Myhre Jensen* 1970) The kidney allograft reaction in non sensitized rabbits is characterized by increasing infiltration by mononuclear cells and swelling or proliferation of glomerular endothelial and mesangial cells during the first days after transplantation followed by proliferation of the vascular endothelium and an exudation of polymorphonuclear cells and fibrin into the glomeruli and blood vessels As a rule the proliferative changes are more widespread than the exudative changes in the glomeruli and vessels Thrombosis and

TABLE 2 *The Frequency (in Percent) of Morphological Alterations in Renal Allografts Removed 1–3 Days after Transplantation from Recipients Sensitized by Donor Kidney Homogenate and from Non Sensitized Rabbits*

	Total	infiltr Cell	Prolif changes glom	Prolif changes vessels	Exudat changes glom	Exudat changes vessels	Thrombosis glom	Thrombosis vessels	Necrosis	chan
Homogenate	17	47	53	0	12	6	12	30	35	[illegible]
Non sensitized	36	48	33	8	8	3	3	19	19	3

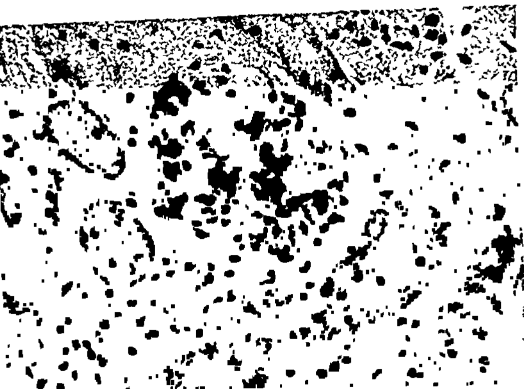


Fig 5 Allograft no 285 (as in Fig 4) Glomerular microthrombosis and cortical necrosis PTAH × 400

accelerated rejection *Dubernard et al* (1968) have demonstrated that the characteristic lesions of allograft rejection can be provoked in the third kidney by injection of serum from a recipient who has experienced an acute allograft rejection

In our experiments, repeated injections of kidney homogenate may have caused a sensitization similar to that obtained by kidney grafting, and the addition of Freund s complete adjuvant may have extended the time of antigenic stimulation The injection of homogenate did provoke some kind of anti body response since cytotoxic antibodies could be demonstrated after but not before the in jections and studies using an immunofluorescent technique showed deposition of IgG in some of the glomerular capillaries and in the intrarenal vessels (*Lund & Sommer Hansen* 1971) Only two allografts in rabbits pre sensitized by kidney homogenate presented a histological pattern similar to that seen in cases of hyperacute rejection Two other grafts showed reactions comparable to the strongest reaction observed in the non-sen sitized rabbits In 14 allografts from rabbits presensitized by kidney homogenates, changes were similar, in extent and severity, to the weakest alterations in non sensitized rabbits It is possible that the homogenate injections in these last mentioned cases induced graft enhancement rather than an acceleration of rejection Enhancement of renal allografts in rabbits has been observed after repeated in jections of donor liver homogenate (*Owen et al* 1968)

In Table 3, the frequency of histological alterations in kidney allografts from rabbits presensitized by kidney homogenate is compared with that in allografts from recipients sensitized by multiple skin grafts (*Lund & Myhre Jensen* 1971) It appears that the frequency of lymphocytic infiltration was about the same in the two groups, proliferative

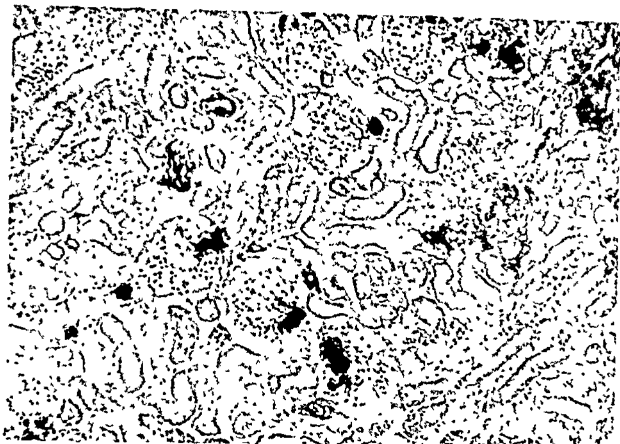


Fig 4 Allograft no 285 removed 4 days after transplantation Glomerular microthrombosis and cortical necrosis PTAH × 100

The increased frequency of proliferative glomerulitis is not surprising since injection of homologous kidney homogenate is known to induce production of nephrotoxic antibodies which—passively transferred with serum into another rabbit—cause membraneous glomerulonephritis and proliferation of glomerular endothelium (*Unanue, Dixon* & *Feldman* 1967) In our experiments, nephrotoxic serum came in direct contact with the specific kidney through transplantation and consequently a similar glomerulonephritis might be expected Thickening of the basement membrane was not observed The observation period was short, however, and the animals studied were young (2–3 months) According to *Beregi* & *Mayersbach* (1969), heterologous nephrotoxic serum mainly provokes a proliferation of glomerular cells in younger animals whereas in older animals thickening of the glomerular basement membrane dominates

Our material is too small to allow any definite conclusions to be drawn concerning the slightly increased frequency of glomerular microthrombosis, but it seems that glomerular microthrombosis is not a conspicuous feature in allografts from animals presensitized by kidney homogenate In this connection it is worth noting that injection of kidney homogenate produces cytotoxic antibodies against donor leucocytes in all cases But neither the level of the antibody titre, nor the presence of cytotoxic antibodies seemed to correlate closely with specific morphological alterations in the graft

A second kidney from the donor providing the primary transplant is frequently rejected within 3 days after transplantation (*Simonsen et al* 1953, *Dempster* 1953) In experiments with rabbits, *Klassen* & *Milgrom* (1969) showed recently that a second kidney from the same donor, as well as a third kidney from another donor, frequently underwent

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CHROMOSOMAL ABERRATIONS AND THEIR RELATION TO MALIGNANCY IN MENINGOMAS: A MENINGOMA WITH RING CHROMOSOMES

JOACHIM MARK

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A benign human tumour, a hypodiploid meningoma, with a ring chromosome in its stemline is described This study, together with previous findings in 11 other benign meningomas, demonstrates that neither considerable aneuploidy nor structural aberrations are reliable cytogenetical indicators of malignant transformation In early *in vitro* passages of the present [illegible] norma [illegible] was se [illegible] shift [illegible] may represent a remnant of a stemline developed at an earlier period of the *in vivo* growth of the meningoma

The chromosomal findings in a recurrent human meningoma were recently reported (*Benedict et al* 1970) Histopathologically, there was no evidence of malignancy in the specimen originally removed The recurrence, however, revealed definite histological malignant changes On both occasions the stemline was hypodiploid, S = 38 and S = 39, respectively, with two to three markers On the basis of these results it was proposed "that malignancy should be seriously considered when significant aneuploidy and marker chromosomes are found in cells from solid tumours, whatever the histological findings are This hypothesis will be discussed in relation to the chromosomal findings in 11 human meningomas previously studied (*Mark* 1969, 1970b), and in relation to a newly detected meningoma with a ring chromosome in its stemline This last tumour was studied in several fixations from material explanted in vitro, and attention was also paid to the chromosomal changes during early *in vitro* passages

MATERIAL AND METHODS

A meningoma, measuring approx 6.0 × 2.5 × 2.5 cm and located in the right parietal region was completely removed from a 65-year-old man, who had suffered from jacksonian seizures for one month prior to the operation Histopathologically the tumour was of the syncytial type (*Russel & Rubinstein* 1963) The tumour cells, arranged in sheets, showed slight nuclear pleomorphism Mi

Received 31 viii 70

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glomerulitis was slightly more frequent in rabbits sensitized by kidney homogenate, endarteritis, vasculitis, exudative glomerulitis and thrombosis were more frequent in the skin sensitized rabbits These observations point to a difference in the effect of sensitization by donor skin and by donor kidney homogenate of the renal allograft in the rabbit

By way of conclusion it should be mentioned that administration of donor kidney homogenate prior to kidney transplantation induces an acceleration of proliferative glomerulitis in renal allografts in rabbits Allograft thrombosis, including glomerular microthrombasis, and necrosis occurs more frequently in presensitized than in non sensitized rabbits, but not as frequently as in recipients presensitized by multiple skin grafts Cytotoxic antibodies could be demonstrated in all sensitized recipients just before transplantation Only two allografts, however, presented a histological pattern characteristic of hyperacute rejection

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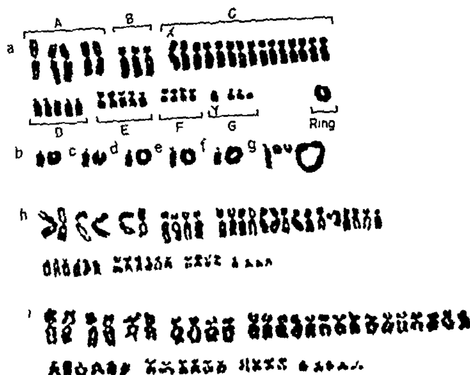


Fig 3 Fixation 1 a Karyotype of a stemline cell S ≈ 42 b–f The biggest D chromosome and the ... see differ ... ith mono-

The results of the karyotype analyses are shown in Table 1 In the present fixation there were 24 different karyotypes among the 51 cells studied Among the 23 42-chromosome cells analysed, 19 had the same karyotype the stemline karyotype (Figs 2a b and 3a Since the 42 chromosome cells were random samples the frequency of S cells in the tumour cell population can be calculated to be about 39 per cent Six of the karyotyped cells had the s number, i e 45 Since all of them had the same karyotype (Fig 3h) the frequency of s-cells in the tumour cell population can be estimated to about 12 per cent

The most outstanding feature of the stem line karyotype was the presence of a fairly big ring chromosome (Figs 2a, b and 3a f) The same marker was also seen in the variant cells with 42 chromosomes in all hypomodal cells, in a few hypermodal cells, and usually duplicated in the triploid and in most of the hypotetraploid cells All metaphases counted were scrutinized for the occurrence of ring chromosomes but none could be found in the cells with 44, 45 and 46 chromosomes, nor in their doubling products Thus, the marker permitted the identification of two different cell populations, each with a small fraction of doubling products The stemline population, with one or rarely two to three ring chromosomes accounted for about 75 per cent of the cells studied (Fig 1, hatched areas), the other group, the sideline population without ring chromosomes, accounted for the remaining quarter of the cells. Nineteen variant cells of the stemline population were analysed (Table 1, karyotypes nos 1,

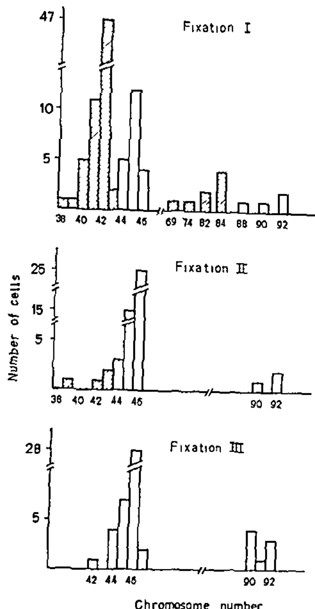


Fig 1 Chromosome counts in three consecutive fixations of the meningoma Hatched areas = metaphases with ring chromosome(s)

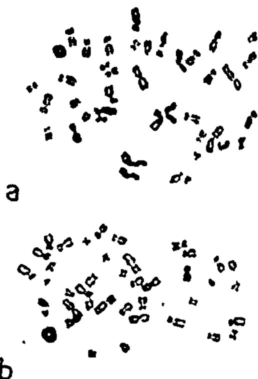


Fig 2a and b Metaphase spreads of stemline cells The ring chromosome is more intensely stained and located in the periphery of the spreads × 1200

toses were extremely rare No malignant features were seen

Fresh pieces of tumour tissue were finely minced with scissors and explanted *in vitro*, as described in detail elsewhere (*Mark* 1969) The chromosomes were studied in three fixations (*I II* and III) made five, 37 and 88 days after explantation, respectively The first fixation was made on material from a primary culture, the second from *in vitro* passage four, and the third from passage nine Methods for chromosome preparation and terminology for marker chromosomes and for the concepts of stemline, S, and sideline(s), s (s_1 s_2 *etc*), are the same as in *Mark* (1969)

RESULTS

Fixation I The first analysis comprised 100 cells The chromosome counts are shown in Fig 1 (top) Almost 50 per cent of all metaphases studied had the stemline number, S = 42 A minor second mode was seen at s = 45, the sideline number The spread from the stemline region including the cells of the sideline was restricted to S ± 4 Twelve per cent of the cells had chromosome numbers in the triploid-tetraploid region There was a predominance of hypotetraploid elements, and in this zone doubling products of the S formed a small mode at 84 Because of the excellent quality of the fixation, practically every metaphase was countable Thus, the frequency of polyploid cells, 12 per cent, determined in the counts should be representative of the mitosing population This also applied to the findings in fixations *II* and III

o the Diploid Male Karyotype in All Except 17 20 and 28 29 Which Are in Relation to the
lale Karyotype

roups		Markers			2n	Number of cells in fixation		
Y	G 21 22	Ring	Small t	Big t		I	II	III
	—3	+1	—	—	38	1	—	—
	—2	+1	—	—	39	—	1	—
—	—1	+1	—	—	40	1	—	—
	—3	+1	—	—	40	1	—	—
	2	+2	—	—	40	1	—	—
—	—1	+1	—	—	41	1	—	—
—	—1	+1	—	—	41	1	—	—
—	—2	+1	—	—	41	1	—	—
—	—1	+1	—	+1	41	1	—	—
	—1	+1	+1	—	41	1	—	—
	—3	+1	+1	—	41	1	—	—
	1	+1	—	—	42	19	1	—
—	—1	+2	—	—	42	2	—	—
	—1	+1	—	—	42	2	—	—
—	—1	+1	—	—	43	1	1	—
—	1	+1	—	—	43	—	1	—
—	—4	+3	—	—	74	1	—	—
—	—2	+2	—	—	84	1	—	—
	1	+1	—	—	84	1	—	—
—	—2	+2	—	—	84	1	—	—
—	—1	—	—	—	42	—	—	1
—	—1	—	—	—	44	2	1	2
—	—2	—	—	—	44	1	1	—
—	1	—	—	—	45	6	5	4
—	—	—	—	—	46	2	10	10
—	+1	—	—	—	47	—	—	1
—	—	—	+1	—	47	—	—	1
	—2	—	—	—	90	1	1	2
—	—	—	—	—	92	1	1	2
			Total number of karyotyped cells			51	23	23

in the periphery of the spreads in more than 75 per cent of the metaphases (Figs 2a b) probably an effect of the so called γ factor (Östergren 1949)

Seven variant cells of the sideline population were analysed (Table 1 karyotypes nos 22 23 25 28 and 29) Three of these had a normal or doubled normal complement but the others were closely related to the s-cells

As in subsequent fixations the frequency of breakage was low about seven per cent In this respect there were no differences between the stemline population and the sideline population The karyological diversity in the former population however indicated that it was less stable than the latter cell group

Fixation II Fifty cells were studied The chromosome counts are shown in Fig 1 (middle) The hypodiploid stemline found in fixa
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hyp
had increased in prominence. The asymmetrical and negative spread in the stem sideline region involved approximately the same range of chromosome numbers as in fixation I but the number of variant cells was reduced The frequency of doubling products

TABLE I *Survey of Karyotypes in Fixation I, II and III The Given Values Are in Re*
Doubled D

Karyotype no	A			B	C	D	E		Chromo F
	1	2	3	4–5	6 X-12	13–15	16	17–18	19-20
1	—1	—	—	—1	—1	—2	—	—1	—
2	—1	—	—	—1	—	—2	—	—1	—1
3	—1	—	—	—1	—1	—2	—	—1	—
4	—1	—	—	—1	—	—1	—	—1	—
5	—1	—	—	—1	—1	—1	—	—2	—
6	—1	—	—	—1	—1	—1	—	—1	—
7	—1	—	—	—1	—	—2	—	—1	—
8	—1	—	—	—1	—	—1	—	—1	—
9	—1	—	—	—1	—	—2	—	—1	—1
10	—1	—	—	—1	—	—2	—	—1	—1
11	—1	—	—	—1	—	—1	—	—1	—
12	—1	—	—	—1	—	—1	—	—1	—
13	—1	—	—	—1	—	—2	—	—1	—
14	—1	—	—	—1	—1	—1	—	—	—
15	—1	—	—	—	—	—1	—	—1	—
16	—1	—	—	—	—	—2	—	—	—
17	—3	—1	—	—2	—	—3	—1	—5	—2
18	—2	—	—	—2	+2	—3	—1	—2	—
19	—2	—	—	—2	+1	—2	—1	—2	—
20	—2	—	—	2	—	—2	—1	—1	—
21	—	—	—	-	—1	—1	—	—1	—
22	—	—	—		—	—	—	1	—
23	—	—	—	—	—		—	—	—
24	—	—	—	—	—	—	—	—	—
25	—	—	—	—	—	—	—	—	—
26	—	—	—	—	—	—	—	—	—
27	—	—	—	—	—	—	—	—	—
28	—	—	—	—	—	—	—	—	—
29	—	—	—	—	—	—	—	—	—

3–11, 13–15 and 17–20) Those in the modal region were all closely related to the S-cells Three of them showed new marker types Thus, in one cell there was an acrocentric marker (t-type) somewhat bigger than the D chromosomes and in two other cells there was a small t marker, only about half as big as the G chromosomes The polyploid variant cells belonging to the stemline population were rarely strict doubling-products of cells in the modal region, superimposed further deviations were common and, in addition, there was sometimes variation in the actual size of the ring chromosome (Fig 3g) Conversely, the ring chromosome in the near-diploid cells showed an unusual constancy with regard to size and morphology It was always monocentric and somewhat more intensely stained than the other chromosomes (Figs 2a, b and 3a–f) Interlockings between the chromatids of the ring chromosome were not seen The origin of the ring chromosome was uncertain but its size suggested a derivation from the lost A 1 chromosome or possibly from the lost B chromosome However, complicated translocations engaging groups D, E and G could not be excluded as possible mechanisms for its appearance The ring chromosome showed a nonrandom location in the metaphase spreads Thus, it was found

son for the scarcity of tumour stemlines with ring chromosomes is their interference with smooth mitotic function as emphasized by *Levan* (1956a b) In the present meningoma the ring chromosome appeared to be a well stabilized component of the stemline Certainly this must be especially important in a benign tumour with its more restricted growth capacity

Histopathologically there was no evidence of a malignant degeneration in the present meningoma and at operation the tumour was well demarcated from the brain tissue and there was no invasion of other adjacent structures The 11 meningomas reported on earlier (*Mark* 1969 and 1970b d) also showed a clinically and histologically benign character More than half of them had one or sometimes as many as four or five markers in their stemline Thus the findings in the present and in previously analysed meningomas do not support the hypothesis of a strong correlation between the occurrence of markers and malignancy Significant aneuploidy was another karyological feature thought to be an indicator of malignancy in solid tumours The present case and at least two of the 11 meningomas earlier described showed considerable heteroploidy The chromosomal variability in these cases was equal to that seen in malignant human tumours (*Mark* 1970a) These results demonstrate the need for caution when cytogenetical observations are to be transformed into clinical terms This is emphasized further by the chromosomal findings in two other benign human tumour types the neurinomas and the pituitary adenomas (*Mark* 1970d and 1969) Thus one of the four neurinomas studied an acoustic tumour had a pseudodiploid stemline with as many as three different marker chromosomes Among the five pituitary adenomas analysed so far two showed extensive heteroploidy and one of these also showed frequent structural aberrations

The serial fixations of the present meningoma illustrated important aspects of the population dynamics during early *in vitro* growth of tumours The original stemline S = 42 with one ring chromosome and the derived variant cells were gradually replaced by a normal diploid stemline and a persisting hypodiploid sideline s = 45 This sideline, also seen in the first fixation increased in prominence during a short period corresponding to the second fixation but then it seemed to be slowly outgrown in its turn by the diploid stemline Thus in a new milieu the most karyologically deviating cells were also the ones with the most restricted growth capacity A similar observation was made in another meningoma studied during early *in vitro* growth (*Mark* 1970b) In this case a 57 chromosome stemline was outgrown by a pseudodiploid stemline This stemline shift was caused clearly by a differential growth rate of tumour cells In the present case however it was impossible to know whether the normal diploid stemline in the second and third fixation actually represented outgrowing stroma cells or a flare up of a persisting fraction of the original normal diploid stemline It is interesting to speculate on the possibility that the loss of a G chromosome might be the first gross progressional step This is supported by the frequent finding in meningomas of a karyotype deficient in G group chromosomes (*Singer & Zang* 1970 *Mark* 1970b)

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was decreased too Thus only six per cent of the tumour cells had polyploid chromosome numbers and they were found in the restricted tetraploid hypotetraploid zone

Ten different karyotypes were found among the 23 cells analysed (Table 1) All of the ten 46 chromosome cells studied had a normal diploid karyotype (Fig 3i) Accordingly the S cells accounted for 50 per cent of the elements in the mitosing population The five 45 chromosome cells analysed also had the same karyotype, and it agreed with that found in fixation I (Fig 3h) However the frequency of s cells in the cell population had increased from 12 to 30 per cent

A ring chromosome was seen only in four hypomodal variant cells (Fig 1 hatched areas) They were all karyotyped (Table 1 karyotypes nos 2 12 15 and 16) and showed deviations similar to those in the stemline population in fixation I The other four variant cells analysed (karyotypes nos 22 23 28 and 29) were closely related to the stem sideline cells

The chromosomal findings in fixation II demonstrated competition between a normal diploid stemline and a reinforced 45 chromosome sideline persisting from fixation I Only remnants of the original stemline population were left the frequency of these cells had dropped from about 75 per cent to less than 10 per cent

Fixation III This study also comprised 50 cells and the chromosome counts are shown in Fig 1 (bottom) In relation to fixation II the S = 46 had increased slightly in prominence whereas the s = 45 showed a substantial diminution The spread in the modal region was still asymmetrical and negative but the range of chromosome numbers involved was decreased Similar to previous fixation the polyploid cells were found in the narrow zone of 90 to 92 but their frequency was increased to 16 per cent

Eight different koryotypes were seen among the 23 cells analysed (Table 1) As in fixation II the ten 46 chromosome cells studied showed a normal diploid karyotype Thus the S cells accounted for 56 per cent of the elements in the mitosing population The four 45 chromosome cells studied had the same karyotype as in previous fixation but their frequency in the cell population was now reduced to 14 per cent

The nine variant cells studied were all closely related to the stem sideline cells (Table 1, karyotypes nos 21 22 and 26 29) One of the 47 chromosome cells had a small t marker which was about half as big as the G chromosomes

No cell with a ring chromosome was seen Thus the original stemline population had been completely outgrown The decreased frequency of s cells was counterbalanced by an increase of the S cells and of the doubling products of both s and S cells

DISCUSSION

Since the appearance of the first reports on ring chromosomes in malignant human tumours (*Levan* 1956a *Ising* & *Levan* 1957) sporadic occurrences have been detected in a variety of malignant conditions in man for instance in mammary carcinomas (*Ishihara et al* 1963 *Sandberg et al* 1967 *Katayama* & *Masukawa* 1963) ovarian carcinomas (*Sandberg et al* 1967) malignant melanomas (*Miles* 1967) malignant gliomas (*Mark* 1970d) and leukaemias (*Meisner et al* 1970) In this context it is of interest to note that ring chromosomes were seen more frequently in metastatic lesions usually associated with effusions than in primary solid tumours Although ring chromosomes have been found also in normal persons this feature indicates a correlation between the occurrence of ring chromosomes and advanced tumour progression *i e* more malignant states In view of this relation the present meningoma is an exception and it is the first benign neoplasm to show this structural anomaly

Among the various morphological types of marker chromosomes found in human tumour stemlines only double minutes (*Mark* 1970c) and possibly also dicentrics are seen more seldom than ring chromosomes A rea

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MALLORY BODIES IN LIVER BIOPSIES WITH FATTY CHANGES BUT NO CIRRHOSIS

A Morphological, Biochemical and Clinical Investigation

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A comparison of histologic and biochemical parametres between 16 *patients with fatty* change of the liver and Mallory bodies and 67 patients with fatty change without Mallory bodies is performed Greater activity expressed by the number of liver-cell necroses, degree of inflammatory reaction, fibrosis of the parenchyma, and transaminase elevation is found in the group with Mallory bodies The importance of Mallory *bodies for the development of fatty change into cirrhosis is discussed*, and the importance of a follow-up investigation is stressed

Prolonged periods of fatty change of the liver are in some cases followed by cirrhosis (*Datu & Culpepper* 1948 *Leevy* 1962), while in other instances even severe steatosis may be present for years without development of cirrhosis (*Buck 1948, Palmer* 1967) Whether the fat itself leads to development of cirrhosis, or whether hepatic necrosis must occur for cirrhosis to develop remains uncertain but most observers require a link between steatosis and fibrosis eventually leading to cirrhosis (*Popper et al* 1955) Hepatocellular changes known as Mallory bodies have been considered to be such links (*Zimmermann* 1955 *Hazinosuta* 1967)

With the purpose of examining whether there are other morphological changes that separate liver biopsies with fatty change and Mallory bodies from liver biopsies with fatty change but without Mallory bodies, we have made a comparison between two series of liver biopsies both with fatty change as the chief morphological diagnosis, one with and one without Mallory bodies In addition a comparison between a series of clinical and laboratory parametres from the corresponding groups of patients has been performed

Received 29 viii 70

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MATERIAL AND METHODS

The mater[illegible] cirrhosis, signs of cholestasis of chlorpromazine type or extrahepatic cholestasis, signs of viral hepatitis, malformations, neoplasm, and vascular disorders are excluded

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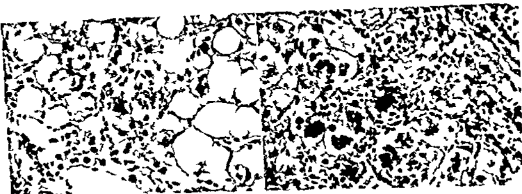


Fig 2 Solitary liver cell containing Mallory body The liver cell is enlarged vacuolized with indistinct borders and is surrounded by neutrophils × 224

Fig 3 Group of liver cells with changes as Fig 2 × 224

Liver cells containing Mallory bodies occur focally singly or in small groups and may be found in all areas of the lobule but are however most frequent in the centrilobular and peripheral areas

Nine out of the sixteen biopsies in group 1 contain few Mallory bodies in five there is a moderate number while only two contain numerous

Sometimes liver cells exhibit changes as seen in necrosis in the form of indistinct cell boundaries karyorrhexis and infiltration with neutrophils in the surroundings (alcoholic hepatitis *Christoffersen et al* 1970)

The changes described in alcoholic hepatitis may affect solitary liver cells containing Mallory bodies (Fig 2) a single cell in a larger group of cells with Mallory bodies or all the cells in the group (Fig 3) Eleven (69 per cent) of the biopsies in group 1 exhibit changes as seen in alcoholic hepatitis

Comparison of the histologic features in the two groups exhibits a number of significant differences which are given in Table 1

Lipogranulomas are found in 14 biopsies from group 1 and in 43 from group 2 In group 1 the lipogranulomas exclusively belong to types 1 and 2 while four biopsies from group 2 contain lipogranulomas type 3 (*Christoffersen et al* 1971)

The inflammatory infiltrates in the parenchyma in group 1 contain lymphocytes in fourteen cases neutrophils in eleven cases plasma cells in three cases and eosinophils in one case In group 2 there is apart from lymphocytes only one biopsy with eosinophils Even when neutrophils which form part of the alcoholic hepatitis in 11 of the biopsies from group 1 are disregarded there are still relatively more biopsies with parenchymal inflammation in the form of lymphocytes in group 1 (14 (87 per cent)) than in group 2 (18 (27 per cent))

Parenchymal fibrosis predominantly centrilobular and/or peripheral in distribution is demonstrated in nine out of the sixteen biopsies in group 1 and in sixteen in group 2 In eight of the nine biopsies with parenchymal fibrosis in group 1 changes as seen in alcoholic hepatitis are also present

There is no statistically significant difference between the two groups as regards the incidence of lytic liver cell necroses variation in size of liver cells and nuclei Kupffer cell proliferation bile duct proliferation as well as content of iron and lipofuscin More than three fourths of all biopsies from both groups exhibit a few lytic liver cell necroses

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as regards Kupffer cell proliferation Bile duct proliferation is demonstrated in a few biopsies and three biopsies (two from group

Sixteen (*group 1*) of the 83 biopsies contain Mallory bodies and have been selected as consecutive biopsies with fatty change and Mallory bodies from a total of 1,100 consecutive liver biopsies (*Christoffersen* 1970*)

The remaining 67 biopsies in the material (*group 2*) are from 67 consecutive patients whose liver biopsy exhibited fatty change without Mallory bodies (*Christoffersen et al* 1971)

The liver biopsies had been performed when there was clinical suspicion of liver disease

The assessment has been performed without any knowledge of the clinical or biochemical data on haematoxylin and eosin and van Gieson Hansen stained sections In nearly all cases additional sections stained for reticulum fibres (*Gomori* 1937) iron (*Perls 1867*) and plasma cells (*Brachet* 1942) have been available

Mallory bodies have been quantified *in the following manner*

\+ the biopsy contains Mallory bodies but on an average not more than one in each lobule

\+ + on an average more than one Mallory body in each lobule but less than five

\+ + + on an average more than five Mallory bodies in each lobule

In addition to quantifying Mallory bodies it has been noted whether alcoholic hepatitis (*Christoffersen et al* 1970), focal lytic necroses (liver cell necroses exclusive of necroses in alcohol hepatitis and lipogranulomas), acidophil bodies lipogranulomas (*Christoffersen et al* 1971) Kupffer cell proliferation inflammation and fibrosis of the parenchyma cholestasis, as well as portal inflammation and fibrosis bile duct proliferation and deposits of iron and lipofuscin in liver cells and Kupffer cells occur Cholestasis is registered when convincing intra—or extracellular bile thrombi are demonstrated When registering Kupffer cell proliferation and other reactions of the mesenchyma elements taking part in the formation of the lipogranulomas have not been included As parenchymal fibrosis stellate smaller or greater areas of collagen fibrils in relation to the central vein or more peripherally situated in the lobule have been registered Furthermore an assessment of the degree of fatty change (1 3) has been performed

As biopsies with lipogranulomas exhibit greater morphological activity (mesenchymal reaction and/or liver cell necrosis) than biopsies without lipogranulomas (*Christoffersen et al* 1971) a possible difference in activity between group 1 and 2 could be due to this condition For this reason a further comparison has been performed between

* One biopsy from the original series has been omitted as it is a repeat biopsy from a patient who already forms part of group 1

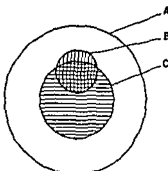


Fig 1 The relation between the incidence of Mallory bodies (circle B 16 patients) and lipogranulomas (circle C 57 patients) in 83 patients with fatty change of the liver (circle A) Expressed in Boole s algebra a comparison (see text) is made between A ∪ B and B (patients with fatty change without and with Mallory bodies) and between B ∩ C and C ∪ B (patients with fatty change with lipogranulomas and Mallory bodies as opposed to patients with fatty change with lipogranulomas and without Mallory bodies)

two subgroups (1A and 2A) which consist of the biopsies from group 1 and 2 that contain lipogranulomas (cf Fig 1)

At the time of biopsy the following biochemical variables were registered Alcaline phosphatase (autoanalysator), serum bilirubin (colorimetric) serum asparate transaminase (Reitman & Frankel s method), prothrombin proconvertin (Owren s method), B S P retention (colorimetric) and serum albumin and gamma globulin (paper electrophoresis)

The consumption of alcohol has been registered and alcoholism is defined as an intake of more than 50 g ethanol/day for more than five years

For the statistic assessment the chi² test has been used for the histologic parametres while the t test following logarithmic transformation for alcaline phosphatase serum asparate transaminase and serum gamma globulin values (*Dixon* 1965) has been utilised for the biochemical The limit for type I—error (2 α) has been set to 0 05

RESULTS

Morphological Findings

The structures which in this material have been registered as Mallory bodies have had the following appearance irregular, frequently horse shoe shaped, finer or coarser hyaline, eosinophilic cytoplasmic masses often situated paranuclearly The liver cells in question are enlarged and most frequently vacuolised with faintly staining cytoplasm but without lipid vacuoles

...psies with Mallory Bodies) and Group 2 (67 Biopsies without Mallory Bodies)

Parenchymal inflammation 0	+	Parenchymal fibrosis 0	+	Portal fibrosis 0	+	Portal inflammation 0	+
2 (13 %)	14 (87 %)	7 (44 %)	9 (56 %)	2 (13 %)	14 (87 %)	0 (0 %)	16 (100 %)
9 (73 %)	18 (27 %)	51 (76 %)	16 (24 %)	44 (66 %)	23 (34 %)	23 (34 %)	44 (66 %)
$p < 0.001$		$p < 0.05$		$p < 0.01$		$p < 0.05$	

...Lipogranulomas and with Mallory Bodies) and Subgroup 2 A (43 Biopsies with Lipo... ...Mallory Bodies)

Parenchymal inflammation 0	+	Parenchymal fibrosis 0	+	Portal fibrosis 0	+	Portal inflammation 0	+
1 (7 %)	13 (93 %)	5 (36 %)	9 (64 %)	1 (7 %)	13 (93 %)	0 (0 %)	14 (100 %)
28 (65 %)	15 (35 %)	30 (70 %)	13 (30 %)	28 (65 %)	15 (35 %)	12 (28 %)	31 (72 %)
$p < 0.001$		$p < 0.05$		$p < 0.001$		$p > 0.30$	

differences as regards sex age or abuse of alcohol

Among the registered laboratory parametres serum asparatetransaminase and BSP retention are elevated in both groups but significantly higher in the group with Mallory bodies Further a significant reduction of serum albumin is found in this group The results of the other laboratory tests are within or just at the limit of the normal range

DISCUSSION

If the histologic activity is expressed by the number of disintegrating liver cells and the inflammatory reaction in the parenchyma there is thus greater activity in the group of biopsies with Mallory bodies than in that without This applies even when necroses and neutrophils which are a part of the alcoholic hepatitis which is found in 69 per cent of the biopsies with Mallory bodies are disregarded

The biochemical activity is likewise greater in groups with Mallory bodies where SGOT is significantly greater It is naturally not possible to decide how great a part of the SGOT elevation that is due to necrosis of liver cells containing Mallory bodies, and how great a part that is due to other types of necrosis

Serial sections have revealed that both acidophil bodies and lymphocytes and plasma cells nearly always are clearly separated from liver cells with Mallory bodies This speaks in favour of Mallory bodies not inducing the

TABLE 1 *The Frequence in the Material of a Series of Histological Qual t es n Gr*

	Acidophil bodies		Lipogranulomas		Degree of fatty change		
	0	+	0	+	+	++	++
Group 1 (16 biopsies with Mallory bodies)	3 (19 %)	13 (81 %)	2 (13 %)	14 (88 %)	2 (13 %)	11 (69 %)	3 (19 %
Group 2 (67 biopsies without Mallory bodies)	55 (82 %)	12 (18 %)	24 (36 %)	43 (64 %)	34 (51 %)	24 (36 %)	9 (13
Differences (χ^2 test)	$p < 0.001$		$0.20 > p > 0.10$		$p < 0.0$[illegible]		

TABLE 2 *The Frequence of a Series of Histological Qualities in Subgroup 1 A (14 B... granulomas bu a*

	Acidophil bodies		Degree of fatty change		
	0	+	+	++	+++
Subgroup 1 A (14 biopsies with lipogranulomas and with Mallory bodies)	2 (14 %)	12 (86 %)	2 (14 %)	9 (64 %)	3 (21 %
Subgroup 2 A (43 biopsies with lipogranulomas and without Mallory bodies)	31 (72 %)	12 (28 %)	18 (42 %)	17 (40 %)	8 (19 %)
Differences (χ^2 test)	$p < 0.001$		$0.10 < p < 0.20$		

1 and one from group 2) exhibit slight cholestasis. All biopsies contain lipofuscin, and approximately one third (from both groups) iron.

Table 2 shows a comparison between the incidence of acidophil bodies, parenchymal inflammation, parenchymal fibrosis, portal fibrosis and portal inflammation and in addition the degree of fatty change in subgroup 1 A (14 biopsies from group 1 with lipogranulomas) and subgroup 2 A (43 biopsies from group 2 with lipogranulomas).

Also comparison of these two subgroups shows a significantly more frequent incidence of acidophil bodies, parenchymal inflammation and parenchymal and portal fibrosis in the group with Mallory bodies, while there is no significant difference as regards to degree of fatty change or the incidence of portal inflammation.

In summary it can thus be stated that there is a statistically significant greater number of biopsies with acidophil bodies, parenchymal inflammation and parenchymal and portal fibrosis in group 1 than in group 2, while there is no significant difference in the incidence of lipogranulomas, lytic liver cell necroses, variation in size of nuclei and cells, portal inflammation, bile duct proliferation, cholestasis, content of iron and lipofuscin or degree of fatty change.

Biochemical and Clinical Findings

The clinical and laboratory parametres are entered in Table 3. There are no significant

Biopsies with Mallory Bod es) and Group 2 (67 Biopsies without Mallory Bodies)

Parenchymal inflammation		Parenchymal fibrosis		Portal fibrosis		Portal inflammation	
)	+	0	+	0	+	0	+
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p < 0 001		p < 0 05		p < 0 01		p < 0 05	

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Parenchymal nflammation		Parenchymal fibrosis		Portal fibrosis		Portal inflammation	
0	+	0	+	0	+	0	+
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p < 0 001		p < 0 05		p < 0 001		p > 0 30	

differences as regards sex age or abuse of alcohol

Among the registered laboratory parametres serum asparatetransaminase and BSP retention are elevated in both groups but significantly higher in the group with Mallory bodies Further a significant reduction of serum albumin is found in this group The results of the other laboratory tests are within or just at the limit of the normal range

DISCUSSION

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Biochemical and Clinical Findings

The clinical and laboratory parametres are entered in Table 3 There are no significant

the contrary, as previously suggested by, among others, *Edmondson et al* (1963) and *Schaffner & Popper* (1970) by necroses of liver cells containing Mallory bodies as a part of the alcoholic hepatitis

A follow up investigation of patients with fatty change and Mallory bodies is in progress and is necessary for a closer evaluation of the possible signification of Mallory bodies for the development of cirrhosis

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TABLE 3 *The Distribution of the Clinical and Laboratory Parametres at the Time of Biopsy in Group with Mallory Bodies (Group 1) and in the Group without Mallory Bodies (Group 2)*

Variable (normal value)	Group 1 Fatty liver with Mallory bodies		Group 2 Fatty liver without Mallory bodies		Significance t test or chi test
	Per cent	Mean ± s d	Per cent	Mean ± s d	
Men	56		70		n s*
Age (years)		60 ± 11		56 ± 12	n s
Alcoholism	75		72		n s
Alkaline phosphatase (< 10 K A units)		10 6 ± 3 8		10 0 ± 4 5	n s§
Serum bilirubin (< 1 0 mg/100 ml)		0 8 ± 0 3		0 9 ± 0 5	n s
Serum asparate transaminase (< 1 7 mmol/1/hr)		3 5 ± 2 0		1 9 ± 1 7	P < 0 05§
Prothrombin Proconvertin (> 60 % of normal)		81 ± 19		87 ± 14	n s
BSP retention (< 5 % after 45 min)		19 ± 9		14 ± 9	P < 0 05
Serum albumin (> 4 4 g/10 ml)		3 44 ± 1 06		4 45 ± 0 74	P < 0 01
Serum γ globulin (< 1 1 g/100 ml)		1 02 ± 0 22		1 10 ± 0 41	n s §
Total number of patients		16		67	-

* Non significant *i e* P > 0 05

§ After logarithmic transformation

development of acidophil bodies and mesenchymal reaction, lymphocytes and plasma cells On the other hand the considerable degree of positive correlation between the incidence of the varibels enumerated above speaks in favour of a temporal and aetiologic connection

In this connection it can be mentioned that Mallory bodies most frequently but not exclusively occur in alcoholics, and that conversely only slightly less than one fourth of the alcoholics in the material have Mallory bodies

In addition to greater activity the group of liver biopsies with Mallory bodies also exhibits more pronounced fibrosis of the parenchyma than the group without Mallory bodies

Has the increased activity any bearing on the development of fibrosis and/or cirrhosis We have in the material presented found disintegration of liver cells in the form of lytic necroses, acidophil bodies lipogranulomas and necroses of liver cells containing Mallory bodies

The follow up investigation renders it probable, that lytic necroses, acidophil bodies and lipogranulomas type 1 and 2 generally disappear without sequaleae and can thus not be the link between fatty change and fibrosis (*Christoffersen et al* 1971) Lipogranulomas type 3 on the other hand seem to give rise to development of connective tissue in the parenchyma (*Christoffersen et al 1971*) As no lipogranulomas type 3 were found in group 1 of the material presented it is not likely that the more pronounced parenchymal fibrosis in this group is due to lipogranulomas but on

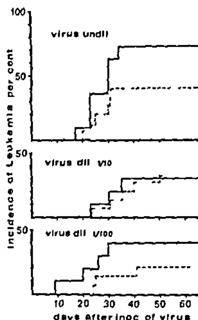


Fig 1 Incidence of leukaemia following intravenous inoculation of myeloid leukaemia virus in various dilutions The latent period to death of the animals in fulminant leukaemia is graphically represented The groups include 10–15 animals each after excluding birds dying from peritonitis within a week after virus inoculation

– – – denotes the pure virus group and ——— denotes the group pre injected with staphylococci

stimulation of the bone marrow by irradiation In the experimental system used in the present investigation it has been shown earlier (*Eckert et al* 1955, *Lagerlof* & *Sundelin* 1963 a) that newly-hatched chicks are most susceptible to virus infection and that they develop a resistance which *increases with age At two months of age* the chicks are completely resistant to virus infection This resistance is probably due to the immunologically mature chicks producing antiviral antibodies (*Lagerlof* & *Sundelin* 1963 b) In the lower age groups, when the chicks are immunologically immature, the higher susceptibility to the virus might also be due to the bone marrow being most cellular at hatching and thus containing relatively more immature target cells This would be in good agreement with the results obtained in the present investigation

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Acta path microbiol scand Section A 79, 208–209, 1971

BRIEF REPORT

ENHANCING THE SUSCEPTIBILITY OF CHICKS TO MYELOID LEUKAEMIA VIRUS BY MYELOID CELL STIMULATION

Bengt Lagerlöf

The possible effect of stimulating the reticulo-endothelial system (RES) on the development of malignant lymphoma has been pointed out by *Murray & Brandt* (1951), *O'Connor* (1961), *Jerusalem* (1968) and *Burkitt* (1969). The inhibitory effect of influenza A I infection on the development of viral mouse leukaemia has been demonstrated by *Libansky & Zavada* (1970). In the experiments to be reported here another system has been used to determine whether stimulation of the target alters susceptibility to leukaemia virus. Fowl leukaemia virus has been inoculated into chicks in which a staphylococcus induced peritonitis had been induced to stimulate the myeloid cell series of the bone marrow, the specific target of the virus. The results obtained show that stimulation of the myeloid cell series apparently increases the susceptibility of chicks to myeloid leukaemia virus.

Experimental

One day old chicks of the Babcock strain purchased from a commercial breeder (Vaderstad Kontrollhonseri) were used. Acute peritonitis was induced by intraperitoneal inoculation of 1×10^{4} staphylococci suspended in saline. Preliminary experiments had shown that this dose produces a severe purulent peritonitis in about 80 per cent of the chicks and is lethal for about 25 per cent. Maximum myeloid response in the bone marrow with an abundance of immature myeloid cells in the bone marrow occurs on the third day after inoculation. Myeloid leukaemia was induced by intravenous inoculation of a plasma preparation of myeloid leukaemia virus (BAI strain) described earlier (*Lagerlöf & Sundelin* 1963 a). Three groups of 20 chicks each received serial dilutions of myeloid virus on the third day after inoculation of the staphylococci. Control groups not pre-injected with staphylococci, were inoculated intravenously with the same dilutions of virus. The latent period to death of leukaemia was recorded. Some of the chicks inoculated with staphylococci died from peritonitis within a week after virus inoculation, i. e. before leukaemia had had time to develop. These chicks were excluded from the final calculations. The final groups include 10 to 15 chicks each.

Results and Comments

The accumulative incidence of leukaemia in the various groups is graphically illustrated in Fig. 1. The groups inoculated with undiluted virus and with virus diluted 1/100 show obvious differences between the staphylococcus inoculated groups and the pure virus groups for both total incidence of leukaemia and latent period to death of the chicks. The latter is most evident in the groups inoculated with virus diluted 1/100. Of the groups inoculated with virus diluted 1/10 the pure virus group has a slightly longer latent period but ultimately a slightly higher incidence of leukaemia than the staphylococcus inoculated group. The difference, however, is not significant.

No difference in the severity of the leukaemia between the staphylococcus virus and the pure virus groups could be found by histological examination of bone marrow, liver, spleen and lungs taken at autopsy.

The results of these studies indicate that stimulation of the target organ promotes the pathogenic effect of leukaemia virus. The results are consistent with earlier studies by *Jerusalem* (1968) and by *Chi & Lagerlöf* (1968) in which the leukaemogenic effect of erythroleukaemia virus was promoted by

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This work was supported by grants from the *Swedish Cancer Society* and *Karolinska Institutets Forskningsfonder.*

Dr. *Kristina Wickman* kindly provided the staphylococcus suspensions.

Efficient technical assistance by mrs *Maud Edenholm* is gratefully acknowledged.

30 amniotic fluid samples were removed immediately before a medical abortion through the abdominal walls, or during the operation through the uterus The stage of gestation has varied from the 14th to the 18th week, and the amniotic fluids have contained 2 to 20×10^3 viable cells/cc Growth medium consisted of 30 per cent foetal calf serum and Medium F 12 (Gibco, N Y) 23 samples gave positive growth sufficient for chromosomal analysis Special attention was paid to the morphology of the cells which grew in the cul ture vessels An epithelial like, ovoid cell type was seen in most of the cultures In a minority of the cultures however, a more fibroblast like cell type was encountered The origin of the foetal cells in the amniotic fluid, which were capable of growing *in vitro* is not known and may not be identical in all cases

Reference 1 *Nadler, H L* J Pediat, 42 912 1968

Therkelsen A J, Philip, J & Bruun Petersen, G, Institute of Medical Microbiology, Aarhus University Aarhus and the Chromosome Laboratory Rigshospitalet Copenhagen EXPERIEN
CES OF PRE... [illegible]

... VITRO

Preliminary results from prenatal determinations of sex and karyotype, and the techniques used to obtain them are presented

For the determination of sex a simple staining with cresylechtviolet is recommended Cultivation of amniotic fluid cells was done in Leighton tubes using Gibco Eagle (BME diploid) with 20 per cent foetal calf serum and 10 per cent human serum as culture medium Cultivation was successful in 33 out of 35 cases (2 samples were lost because of contamination), and usable chromosome preparations were made in 32 of the 33 cases

A case of D/G translocation diagnosed *in utero* is presented

The karyotype was determined 11 days after the amniocentesis, and it is concluded that, with the technique used the karyotype of an embryo can be determined in most cases within 14 days after amniocentesis

Wahlström J Psychiatric Research Center, St Jörgen Hospital and Psychiatric Department III Lillhagen Hospital, University of Gothenburg Gothenburg *VIABILITY OF AMNIOTIC CELLS AT DIFFERENT STAGE OF GESTATION*

53 samples of amniotic fluid were studied and subjected to two different types of examinations First the total number of cells was calculated in Burker chambers up to a volume of 10 mm³ After staining with trypan blue the number of living cells per same volume was calculated, using a modified method described by Pertoft et al (1) The number of living cells per unit of volume apparently increases between the 11th and the 31th week of gestation Thus the best time for amniocentesis for chromosome analysis, i e the period providing the best conditions for culture in view of the contigency of therapeutic abortion, should be between the 15th and 18th week of gestation

Reference 1 *Pertoft, H, Pack, O & Lindahl-Kiessling, Kerstin* Exptl Cell Res, 50 355, 1968

de la Chapelle, A & Schröder, J, The Folkhälsan Institute of Genetics, Helsingfors POSSIBLE ASSESSMENT OF FOETAL SEX IN MAN FROM CELLS IN THE MATERNAL CIRCULATION

In human females pregnant with a male foetus, a small number of XY cells can be found on chromosomal analysis of lymphocytes from the peripheral blood (1, 2) In an attempt to detect such foetal cells without karyotyping hundreds or even thousands of mitoses, use might be made of the fluorescence produced by staining with quinacrine (3), since the differential fluorescence of part of the Y chromosome is detectable in interphase nuclei (4)

To test the sensitivity of the method, cultured male and female lymphocytes were fixed and thereafter mixed in various proportions Quinacrine hydrochloride stained slides of these mixtures were scored for the proportion of nuclei containing Y bodies For each mixture, 1000–2000 cells were examined

The results showed that when 100 per cent of the cells were male, 21 5 per cent of the nuclei contained a fluorescent area fulfilling our criteria for the Y body With 10 per cent male cells and 90 per cent female cells the number of nuclei containing Y bodies was 1 95 per cent, at 1 per cent male cells it was 0 25 per cent and at 0 5 per cent male cells it was 0 30 per cent When only female cells were present no nuclei containing Y bodies were seen It thus appears to be possible to detect male cells in this system if their number is in the order of 0 5 per cent of all the cells in the sample

This method, if proven to give comparable results with lymphocytes from pregnant women, might make it possible to ascertain, in a safe and relatively simple way, the sex of human foetuses Many pitfalls and difficulties are apparent It is not known whether foetal cells always occur in the maternal blood in what proportions they occur at different times or how long they remain there We have initiated a study designed to settle these questions

NORDIC SOCIETY FOR CELL BIOLOGY

TRANSACTIONS OF THE SCANDINAVIAN MICROSYMPOSIUM IN CYTOGENETICS

August 28–29, 1970, Helsinki, Finland

PRENATAL DIAGNOSIS OF GENETIC DISEASE

de Grouchy, J & *Trebuchet C* Clinique de Genetique Medicale, Hôpital des Enfants Malades Paris A SEARCH FOR FOETAL CELLS IN THE MATERNAL BLOOD STREAM

Walknowska et al (1) studied the karyotypes of blood lymphocytes in 30 pregnant women and concluded that foetal lymphocytes are transferred to the mother The diagnosis of a male foetus is possible already at the 14th week of pregnancy The results of a similar study on 21 pregnant women are reported here

Cases 1 8 46 XY cells were found with a variable frequency in the maternal blood In all of these cases the pregnancy terminated in a boy In three instances chromosome markers and other chromosome rearrangements were observed

Cases 9 14 No 46 XY cell was detected, and the pregnancy terminated in a girl Here again breakage and selective endoreduplication were observed Cases 15 17 No 46 XY cell was observed (in 800 cells) and the pregnancy terminated in a boy Cases 18 20 The mothers had previously given birth to a boy In each case one 46 XY cell was found and the pregnancy terminated in a girl Case 21 Two 46 XY cells were found and the mother gave birth to a girl She had admittedly had no previous pregnancy

The diagnosis of a male foetus thus seems possible in a woman who has had no previous male pregnancy The persistence of cells from previous pregnancies is likely

Breakage the permanent background of chromosomal rearrangement and endoreduplication are important pitfalls They produce rare abnormal cells, while the purpose of the study is precisely to discover a particular type of rare cell in a population of cells Partial results from 13 cases have previously been published (2)

References 1 *Walknowska J, Conte F A* & *Grumbach M M* Lancet, *I* 1119, 1969—2 *de Grouchy, J* & *Trebuchet, C* Bull Europ Soc Hum Genet *4* 65, 1970

(Received 14 1 71)

Hahnemann, N Institute of Human Genetics Copenhagen BIOPSY FROM THE FOETAL MEMBRANES AND AMNIOTIC FLUID AS A SOURCE FOR MONOLAYER CULTURE OF FOETAL CELLS

Foetal cells from amniotic fluid, and tissue from foetal membranes are utilized as a tool in foetal genetic diagnosis in early pregnancy Amniotic fluid is obtained by amniocentesis, tissue from the foetal membranes by biopsies through a specially developed hysteroscope Both sources are utilized for the culture of foetal cells Amniocentesis is a safe procedure carried out in pregnancies after the 15th week but neither amniocentesis nor biopsies are today applicable routinely in pregnancies before the 15th to the 16th week The culture of cells obtained by biopsy is a safe procedure while successful culture of cells from amniotic fluid apparently depends on the pregnancy age The problems involved in both amniocentesis and cell culture from the amniotic fluid increase with a decreasing pregnancy age owing to low amniotic fluid volume and cell content especially before the 15th to 16th week

Aula P, Children s Hospital University Central Hospital of Helsinki and III Department of Pathology University of Helsinki Helsinki AMNIOCENTESIS AS A TOOL FOR DIAGNOSIS OF GENETIC DISEASES

Foetal amniotic cells were cultivated according to the methods described by Nadler (1)

The aim of the experiments has been to develop a reliable cell culture procedure to be used in prenatal diagnosis of chromosomal abnormalities

30 amniotic fluid samples were removed immediately before a medical abortion through the abdominal walls or during the operation through the uterus The stage of gestation has varied from the 14th to the 18th week and the amniotic fluids have contained 2 to 20×10^3 viable cells/cc Growth medium consisted of 30 per cent foetal calf serum and Medium F 12 (Gibco N Y) 23 samples gave positive growth sufficient for chromosomal analysis Special attention was paid to the morphology of the cells which grew in the culture vessels An epithelial like ovoid cell type was seen in most of the cultures In a minority of the cultures however a more fibroblast like cell type was encountered The origin of the foetal cells in the amniotic fluid which were capable of growing *in vitro* is not known and may not be identical in all cases

Reference 1 *Nadler H L* J Pediat *42* 912 1968

Therkelsen A J Philip J & Bruun Petersen G Institute of Medical Microbiology Aarhus University Aarhus and the Chromosome Laboratory Rigshosp talet Copenhagen EXPERIENCES OF PRENATAL GENETIC DIAGNOSIS AND PRESENTATION OF A CASE OF D/G TRANSLOCATION DIAGNOSED IN UTERO

Prel minary results from prenatal determinations of sex and karyotype and the techniques used to obtain them are presented

For the determination of sex a simple staining with cresylechtviolet is recommended Cultivation of amn otic flu d cells was done in Leighton tubes us ng "G bco Eagle (BME-diploid) with 20 per cent foetal calf serum and 10 per cent human serum as culture medium Cultivation was successful in 33 out of 35 cases (2 samples were lost because of contamination) and usable chromosome preparations were made in 32 of the 33 cases

A case of D/G translocation diagnosed *in utero* is presented

The karyotype was determined 11 days after the amniocentesis and it is concluded that with the techn que used the karyotype of an embryo can be determ ned in most cases within 14 days after amniocentesis

Wahlstrom J Psychiatric Research Center St Jorgen Hospital and Psychiatric Department III Lillhagen Hosp tal University of Gothenburg Gothenburg *VIABILITY OF AMNIOTIC CELLS AT DIFFERENT STAGE OF GESTATION*

43 samples of amniotic fluid were stud ed and subjected to two d fferent types of examinations First the total number of cells was calculated in Burker chambers up to a volume of 10 mm³ After staining with trypan blue the number of living cells per same volume was calculated using a modified method described by Pertoft et al (1) The number of living cells per unit of volume apparently increases between the 11th and the 34th week of gestation Thus the best time for amniocentesis for chromosome analysis i e the period providing the best conditions for culture in view of the contingency of therapeutic abortion, should be between the 15th and 18th week of gestation

Reference 1 *Pertoft H Back, O & Lindahl Kiessling Kerstin* Exptl Cell Res, *50* 355, 1968

de la Chapelle A & Schroder J, The Folkhalsan Institute of Genetics Helsingfors POSSIBLE ASSESSMENT OF FOETAL SEX IN MAN FROM CELLS IN THE MATERNAL CIRCULATION

In human females pregnant with a male foetus a small number of XY cells can be found on chromosomal analysis of lymphocytes from the peripheral blood (1, 2) In an attempt to detect such foetal cells without karyotyping hundreds or even thousands of mitoses use might be made of the fluorescence produced by staining with quinacrine (3) since the differential fluorescence of part of the Y chromosome is detectable in interphase nuclei (4)

To test the sensitivity of the method cultured male and female lymphocytes were fixed and thereafter mixed in various proportions Quinacrine hydrochloride stained slides of these mixtures were scored for the proportion of nuclei containing Y bodies For each mixture 1000–2000 cells were examined

The results showed that when 100 per cent of the cells were male, 21 5 per cent of the nuclei contained a fluorescent area fulfilling our criteria for the Y body With 10 per cent male cells and 90 per cent female cells the number of nuclei containing Y bodies was 1 95 per cent, at 1 per cent male cells it was 0 25 per cent and at 0 5 per cent male cells it was 0 30 per cent When only female cells were present no nuclei containing Y bodies were seen It thus appears to be possible to detect male cells in this system if their number is in the order of 0 5 per cent of all the cells in the sample

This method if proven to give comparable results with lymphocytes from pregnant women might make it possible to ascertain in a safe and relatively simple way the sex of human foetuses Many pitfalls and difficulties are apparent It is not known whether foetal cells always occur in the maternal blood in what proportions they occur at different times or how long they remain there We have initiated a study designed to settle these questions

Acta path microbiol scand Section A 79, 210-216, 1971

NORDIC SOCIETY FOR CELL BIOLOGY

TRANSACTIONS OF THE SCANDINAVIAN MICROSYMPOSIUM IN CYTOGENETICS

August 28–29, 1970, Helsinki, Finland

PRENATAL DIAGNOSIS OF GENETIC DISEASE

de Grouchy, J & Trebuchet, C, Clinique de Génétique Médicale, Hôpital des Enfants Malades, Paris A SEARCH FOR FOETAL CELLS IN THE MATERNAL BLOOD STREAM

Walknowska et al (1) studied the karyotypes of blood lymphocytes in 30 pregnant women and concluded that foetal lymphocytes are transferred to the mother The diagnosis of a male foetus is possible already at the 11th week of pregnancy The results of a similar study on 21 pregnant women are reported here

Cases 1 8 46, XY cells were found with a variable frequency in the maternal blood In all of these cases the pregnancy terminated in a boy In three instances, chromosome markers and other chromosome rearrangements were observed

Cases 9 14 No 46, XY cell was detected, and the pregnancy terminated in a girl Here again breakage and selective endoreduplication were observed Cases 15–17 No 46, XY cell was observed (in 800 cells) and the pregnancy terminated in a boy Cases 18 20 The mothers had previously given birth to a boy In each case, one 46, XY cell was found and the pregnancy terminated in a girl Case 21 Two 46, XY cells were found and the mother gave birth to a girl She had admittedly had no previous pregnancy

The diagnosis of a male foetus thus seems possible in a woman who has had no previous male pregnancy The persistence of cells from previous pregnancies is likely

Breakage, the permanent background of chromosomal rearrangement, and endoreduplication are important pitfalls They produce rare abnormal cells, while the purpose of the study is precisely to discover a particular type of rare cell in a population of cells Partial results from 13 cases have previously been published (2)

References 1 *Walknowska, J, Conte, F A & Grumbach, M M* Lancet, 1 1119, 1969–2 *de Grouchy, J & Trebuchet C* Bull Europ Soc Hum Genet 4 65, 1970

Hahnemann, N, Institute of Human Genetics Copenhagen BIOPSY FROM THE FOETAL MEMBRANES AND AMNIOTIC FLUID AS A SOURCE FOR MONOLAYER CULTURE OF FOETAL CELLS

Foetal cells from amniotic fluid, and tissue from foetal membranes are utilized as a tool in foetal genetic diagnosis in early pregnancy Amniotic fluid is obtained by amniocentesis, tissue from the foetal membranes by biopsies through a specially developed hysteroscope Both sources are utilized for the culture of foetal cells Amniocentesis is a safe procedure carried out in pregnancies after the 15th week, but neither amniocentesis nor biopsies are today applicable routinely in pregnancies before the 15th to the 16th week The culture of cells obtained by biopsy is a safe procedure, while successful culture of cells from amniotic fluid apparently depends on the pregnancy age The problems involved in both amniocentesis and cell culture from the amniotic fluid, increase with a decreasing pregnancy age, owing to low amniotic fluid volume and cell content, especially before the 15th to 16th week

Aula, P, Children's Hospital University Central Hospital of Helsinki and III Department of Pathology University of Helsinki, Helsinki AMNIOCENTESIS AS A TOOL FOR DIAGNOSIS OF GENETIC DISEASES

Foetal amniotic cells were cultivated according to the methods described by Nadler (1)

The aim of the experiments has been to develop a reliable cell culture procedure to be used in prenatal diagnosis of chromosomal abnormalities

(Received 11 1 71)

The effect seems to be induced by the metaphase nucleus included in a syncytium with an interphase nucleus

Haapala O K, Department of Genetics University of Turku Turku SPREADING TECHNIQUE AS A MOUNTING METHOD FOR ELECTRON MICROSCOPY OF CHROMOSOME FIBRES

The organization of eukaryote chromosome fibres is obviously more normal in thin sectioned cells fixed with protein immobilizing buffered aldehyde, or water phase removing methyl alcohol than in a chromosome mounted directly on a specimen grid Several investigations were made with Drosophila sperm and fungal spores in order to devise a reliable method of demonstrating the molecular structure of nucleohistone fibres and the alterations induced in this structure by chemical agents For this purpose the spreading technique described by So... [illegible]

[illegible]

ed by electron micrographs apparent changes in the fibre organization took place during *air-drying* and dehydration after spreading the material on a water surface Air-drying before staining produced unspecific aggregates of fibres and a coarse fibrillar network was observed in dehydrated preparations This could have resulted *from the collapse* of fibres after removal of water by ethanol The fibrillar organization of chromosomes was retained if samples were stained immediately *after spreading* and dried without dehydration

Reference 1 *Solari A J* Proc Natl Acad Sci U S 53 503 1965

Schult Larsen J Bang J & Northeved A, Institute of Medical Genetics University of Copenhagen A SEMI AUTOMATIC METHOD FOR ANALYSIS OF CHROMOSOME PREPARATIONS OF HUMAN CELLS

In the field of human cytogenetics the extremely time consuming work involved in analysis represents a *factor which* restricts severely further development If the number of cells to be *analysed* were considerably increased it might be possible to solve many h... [illegible]

fol [illegible]

m [illegible] ... of analysis have proved rather disappointing 8 to 10 per cent of the chromosomes being incorrectly recorded In the present study the object has been to consider the *problems* from a simplified point of view The basic idea is to have only two parameters per chromosome determined viz relative size and shape It is explained how a pencil follower may serve for this purpose subsequently a computer is used to copy, as far as possible the visual analysis technique hitherto applied in the field of human genetics

Hayry P & Virolainen M, Laboratory of Immunology, III Department of Pathology and IV Department of Surgery, University of Helsinki, Helsinki METHOD FOR CHROMOSOME PREPARATION FROM MOUSE BLOOD LYMPHOCYTES

The existence of certain chromosome markers in some inbred mouse strains makes cytogenetic analysis of the mouse a useful tool We report a method (1) which gives large numbers of analysable metaphase plates per animal without *the need of* sacrificing it A blood sample of *approximately* 0 5 ml is drawn from the animal through a heart puncture *or through an orbital* plexus bleeding into a heparinized syringe (Heparin 5000 IU/ml 1 10) The red cells [illegible] equal vo[illegible] ly Franc[illegible] *right position* If necessary the leucocyte yield can be *increased ten-fold* by injecting the test animal three days prior to the bleeding with 0 1 ml of 1 3–4 diluted supernatant fluid from *B pertussis* culture The buffy coat cells are separated and washed twice with phosphate buffered saline *They* are suspended in a concentration of 1 0–1.5 × 10^6 lymphocytes/ml *to a* culture medium *consisting of* double strength Eagle s amino acids in Earle s balanced salt solution 10 per cent of foetal calf serum and streptomycin (100 *µg*/ml) — penicillin (100 IU/ml) Phytohaemagglutinin (PHA M, *Difco Laboratories* Detroit Mich) is added in an optimal concen*tration of 1 150 The* cell *suspension* is distributed in one ml quantities into sterile loosely stoppered round bottom Wassermann tubes The tubes are incubated in an *upright position* in a humidified atmosphere of 5 per cent CO_2 and air at 37°C *for appr 70 hours Thereafter* 0 1 *µg* of Colcemid® (Ciba Pharmaceuticals, Summit, N J) is added The cultures are harvested 2–4 hours *later 4 ml of* distilled water is added and the tubes are gently shaken After 10 *minutes'* hypotonic treatment the procedure of *Moorhead et al* (2) is followed Usually *one or two cultures can* be established from one non pertussis treated animal and there are approximately *100–300* analysable metaphase plates per culture Pretreatment of the test animal with pertussis culture *fluid* increases *the yield* up to ten fold We have applied this technique to eight different mouse strains and it has been successful with all the strains tested

References 1 *Walknouska, J, Conte, F A & Grumbach, M M* Lancet, *1* 1119, 1969 —2 *de Grouchy, J & Trebuchet, C* Bull Europ Soc Hum Genet, *4* 65, 1970 —3 *Caspersson, T, Zech, L, Johansson, C & Modest, E J* Chromosoma, *30* 215, 1970 —4 *Pearson, P L, Bobrow M & Vosa, C G* Nature, *226* 78, 1970

FREE COMMUNICATIONS

Zech, L, Institute for Medical Cell Research and Genetics, Medical Nobelinstitute, Karolinska Institutet, Stockholm QUINACRINE MUSTARD FOR DETERMINATION OF GENETIC SEX AND CHROMOSOME ANALYSIS

Human chromosomes treated with the alkylating agent quinacrine mustard (QM) bind the dye differentially The most prominent fluorescence is exhibited by the distal part of the long arm of the male Y chromosome (1, 3) This fluorescent region persists during interphase as a heteropycnotic fluorescent body (1) which can be useful for determining genetic sex in resting nuclei In addition, strongly fluorescent regions from other chromosomes persist during interphase. Cases have been described in which these bodies are as large as the fluorescent part of the Y chromosome These findings make it necessary to combine the QM-technique with other techniques, for instance analysis of the Barr body, or preferably an analysis of the fluorescence pattern of a metaphase of the same individual for precise identification of genetic sex In addition to the highly fluorescent regions on same metaphase chromosomes, a fine pattern of different fluorescent intensities along each chromosome has been found This pattern is characteristic for each individual chromosome, and can therefore be used for identification purposes

References 1 *Caspersson, T, Zech, L & Johansson, C* Exptl Cell Res, *60* 315, 1970 —2 *Caspersson, T, Zech, L, Johansson, C & Modest, E J* Chromosoma *30* 215, 1970 —3 *Zech, L* Exptl Cell Res, *58* 463, 1969

Carlsson, S A, Ringertz, N R & Savage, R E, Institute for Medical Cell Research and Genetics, Medical Nobelinstitute, Karolinska Institutet, Stockholm FUSION OF RAT MYOGENIC CELLS WITH DIFFERENTIATED CHICK CELLS

During normal myogenesis mononucleated myoblasts fuse to form myotubes In these cells contractile proteins form and aggregate to myofibrils The whole process can be reproduced *in vitro* by allowing embryonic muscle cells to grow and fuse on collagen coated glass slides In these cultures myoblasts can easily be distinguished from epithelial cells and fibroblasts which also proliferate Only myoblasts seem to take part in the fusion process If rat myoblasts are co cultured with chick myoblasts, the myotubes will contain both rat and chick nuclei within the same cytoplasm Using UV-inactivated Sendai virus, as described by Harris and co workers, it is possible to fuse myogenic cells also with other cell types We have used this technique to produce rat myoblast × chick erythrocyte and rat myoblast × chick fibroblast hybrids, in addition to the spontaneously forming rat myoblast × chick myoblast hybrid cells The chick erythrocyte nucleus, which normally does not synthesize DNA or RNA, is reactivated in myoblast and myotube cytoplasm The synthesis of both types of nucleic acids is resumed in the chick nucleus at the same time as the nucleus grows in size and the chromatin becomes more dispersed At later stages nucleoli form Since the different types of chick nuclei thus become or remain active in the rat myoblast and myotube cytoplasm, it is of interest to map which chick genes are expressed By immunizing rabbits with chick myosin antibodies have been obtained, which after absorption with rat muscle, are specific for chick myosin These antibodies are presently used as a tool in examining whether the three different types of cell hybrids involving chick nuclei will code for this luxury molecule"

Stenman, S, III Department of Pathology, University of Helsinki, Helsinki DNA SYNTHESIS IN PULVERIZED HELA CELL NUCLEI

Chromosome pulverization has been suggested to be due to a precocious condensation of chromatin (PCC) in interphase nuclei fused with a metaphase cell We have used betapropionlactone inactivated Sendai virus as a cell fusing agent The incorporation of tritiated thymidine into morphologically different kinds of PCC was investigated By pulse labelling with tritiated thymidine in HeLa cells, partially synchronized with excess thymidine, it was found that PCC which formed a completely irregular 'pulverized' mass of chromatin stemmed from the S phase On the other hand, PCC forming slender strands resembling pro or prometaphase chromatin stemmed from G 2 or from the very end of the S

The results are compatible with the hypothesis that PCC is a condensation of interphase chromatin, apparently not yet mature to enter mitosis

Hannah Ala a A & Vunikka Y Department of Genetics University of Turku Turku CYTOCHEMISTRY OF THE NUCLEOLUS ORGANIZER IN SALIVARY GLAND CHROMOSOMES OF *DROSOPHILA MELANOGASTER*

Comparisons were made between the organization and staining react on of the nucleolus and the bands in the chromosomes of salivary gland cells from *Drosophila melanogaster*, by means of the Feulgen reaction or treatment with dyes considered to be specific either for DNA or RNA These include methyl green and pyronine G toluidine blue Azur B and light green and orange G with aceto-orcein The most satisfactory results were obta ned with Azur B using a concentration of 1 mg ml Azur B buffered to pH 4 0, and staining for 2 3 hours at 40°C The nucleolus in Azur B preparations was purplish pink and contained granular and filamentous structures which were either blue or purple in colour The banding pattern of the chromosomes did not differ significantly from that of the Bridges maps and the majority of the bands were blue in colour The major disadvantages i e that the cytoplasm becomes a dark purple reticular mass often obscuring the chromosomes was partially overcome by a brief treatment of the glands with 1N HCl prior to fixation. This short pretreatment with HCl also results in the partial loss of the ribonuleoprotein component of the nucleolus It leaves a purplish pink stained loop or back bone contiguous with the chromosome and possibly comprised primarily of the DNA responsible for the transcription of the ribosomal RNA

Ahn trom G Institute of Biochemistry University of Stockholm Stockholm REPAIR OF RADIATION DAMAGE IN HIGHER ORGANISMS

Rest ng plant seeds are highly resistant to gamma rad at on compared with the metabolizing tissue of the same organism For example 50 krads of gamma rays cause the same damage to resting barley seeds as 1 krad given to seeds pre soaked in water for 12 15 hours For neutron irradiated seeds the correspond ng dose values are 1 and 0 25 krad respectively Neutrons are biologically more effect ve than gamma rays and less influenced by the metabolic stage

If caffeine a subs ance which is believed to interfere with repair processes in irrad ated cells is present during the first hours of germination the damage caused by gamma rays is potentiated by a factor of 2 Caffeine appl ed after the irradiated seeds have germinated for 5 hours in water has no effect indicating that the repair processes have been completed within this time On the other hand caffeine has no influence on neutron induced damage Neutron induced lesions seem therefore to be qualitatively different from lesions induced by gamma rays

Th s phenomenon could be explained if we assume that the important lesions caused by gamma rays are mainly single strand breaks in the DNA which can be repaired while neutrons give a high proportion of double strand breaks constituting an irrepairable damage For several reasons, however, this explanation does not seem to be true Caffeine has little effect on the joining of single strand breaks in irradiated hamster cells On the chromosomal level neutron irradiation should also lead to more incomplete aberrations than gamma irradiation if the broken chromosomes cannot rejoin However this does not seem to be the case A more probable explanation can be based on the difference in spatial distribution of lesions neutron induced les ons being formed practically in contact with each other in the track of the ionizing particle while gamma induced lesions are produced at a distance from each other This will influence the ratio repair misrepair and also how this ratio can be affected by various treatments after radiation

Edgren J The Folkhälsan Institute of Genetics Helsingfors THE EFFECT OF CYS[illegible] ON CHROM[illegible] BY RAD[illegible] CYTES IN VITRO

The purpose of the present project was to devise a method [illegible] cytes in [illegible] radiation [illegible]

The experiments were performed with fresh whole blood from healthy donors and cysteine was used as the protective substance After treatment of the cells with 0 5 1 or 5 mg/ml cysteine and irradiation with 0–1000 rads the cells were cultivated for 48 and 72 hours and preparations made for chromosomal analysis

The following parameters were analysed the frequency of [illegible] lyploid cell [illegible] the number [illegible] ficant corr[illegible] was shown to exist between the elevation of the dose level and the frequency of the different types of aberrations studied at the dose range of 0–500 rads At 1000 rads severe destruction of the cells made analysis of the chromosomes unreliable

A radioprotective effect was demonstrated after treatment of the cells with 5 mg/ml cysteine before irradiation This effect was seen in the oc

References 1 *Hayry, P, Virolainen, M & Defendi V* Proc Soc Exptl Biol Med, *133* 637, 1970 —2 *Moorhead P S, Nowell, P C, Mellman, W J, Battips, D M & Hungerford, D A* Exp Cell Res *20* 613, 1960

Hansen Melander, E & Melander, Y, Institute of Genetics University of Lund Lund HAPLOID DIPLOID MOSAIC IN RABBIT EMBRYO

In a litter of 8 rabbit blastocysts one specimen was found to be a mosaic of haploid and diploid cells This embryo was considerably retarded in growth, corresponding in size to a blastocyst 110-115 hours p c The embryo consisted of about 3550 cells Of these 550 came from the inner cell mass

The mitotic index in the cells of the embryonic disc was significantly higher than that in the cells of the trophoblast The percentage of haploid cells was 85 for the whole embryo The chromosome number of the haploid cells was $n = 22$, with a complete group 7–12, the X chromosome being one of the pairs 8–10 (Nichols et al 1965) The diploid cells had $2n = 44$, with a chromosome complement equal to that of a diploid female cell in somatic tissue Two diploid triploid mosaics of the same age were encountered in litters from two different does

Reference *Nichols, W W, Levan A, Hansen Melander, E & Melander Y* Hereditas *53* 63 76, 1965

Gripenberg Ulla, Elfving, J & Gripenberg L Department of Genetics and Aurora Hospital Helsinki A CASE OF MONOSOMY G?

A preliminary report is given of a child with monosomy G diagnosed in the blood cells The patient showed several minor abnormalities and a somewhat retarded development during early infancy Her mental development appeared normal at the age of 10 months

CHROMATIN STRUCTURE AND CHROMOSOME DAMAGE

Natarajan, A T, Institute of Radiobiology, University of Stockholm, Stockholm DIFFERENTIAL RESPONSE OF FUNCTIONAL AND

The distribution of chromatid aberrations induced by Mitomycin C among the individual chromosomes of female and male Chinese hamster cells was studied *in vitro* The aberrations were found to be non randomly distributed Among the autosomes, the chromosomes possessing structural heterochromatin were often involved in aberrations as well as in homologous exchanges The inactivated X chromosome in the female cells offers a situation where the short arm is functionally heterochromatic and the long arm structurally heterochromatic, thus enabling an analysis of their response for aberration formation 96 per cent of the aberrations involved the structural heterochromatin while 4 per cent involved the functional heterochromatin The long arm of the inactivated X was affected more often (5 to 10 times) than the long arm of the functional X though both are structurally heterochromatic The possible roles of (a) structure of heterochromatin (b) the chromocentre formation and their association (c) allocycly and (d) the qualitative differences in the DNA of different types of heterochromatin are discussed in relation to the formation of chromatid aberrations

Leisti, J, Children's Hospital University Central Hospital of Helsinki Helsinki OBSERVATIONS ON SECONDARY CONSTRICTIONS IN MITOTIC HUMAN CHROMOSOMES

Five families with a prominent paracentric secondary constriction of chromosome No 9 were studied The marker constriction was present in all analysed leucocyte metaphase it was hereditary in nature and was regarded as a normal structural variant

The short arm the secondary constriction and the euchromatic distal segment of the long arm of chromosomes No 9 were measured in from 6 to 10 cells from each of the 18 family members The means were calculated separately for the two homologues and the results were subjected to statistical analysis It could be shown that 1) the lengths of the euchromatic segments of chromosome No 9 were stable and showed no difference between the homologues 2) the length of the secondary constriction was stable for each chromosome No 9 but could differ very significantly from that of its homologue and that 3) the size differences of the chromosomes No 9 were caused by the size differences of the secondary constrictions It was suggested that the chromosome No 9 should not be identified on the basis of absolute size or centromeric index but rather on the basis of the presence of a paracentric secondary constriction

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IDIOPATHIC PULMONARY HYPERTENSION IN INFANCY AND CHILDHOOD

Microangiographic and Histological Observations in Five Cases

BENGT ROBERTSON

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Five autopsy cases of idiopathic pulmonary hypertension (IPH) in infancy and childhood are reported In two cases, the diagnosis had been verified at cardiac catheterization, whereas in the remaining three cases the diagnosis was based upon indirect clinical and morphological evidence The pulmonary vasculature was studied by microangiographic and histological techniques, including morphometry of muscular pulmonary arteries In two neonatal cases (ages 5 days 16 days), there was tortuosity of intralobular pulmonary arteries and carry-over of the foetal type of thick walled muscular pulmonary arteries and arterioles with moderate superimposed medial hypertrophy In one subject aged 8 months, muscular and transitional pulmonary arteries showed considerable medial hypertrophy, but no obliterative features The increase of medial mass in muscular pulmonary arteries and the absence of obliterative vascular lesions suggest that a vasospastic mechanism was responsible for the pulmonary hypertension in these three cases In the two cases of older patients (ages 1 year and 10 months, 10 years) the microangiograms and the lung sections displayed a more complex pattern of severe vascular lesions medial hypertrophy in muscular pulmonary arteries, obliterative intimal fibrosis arterial necrosis and plexiform lesions These vascular changes are identical to those found in cases of IPH in adult patients, as well as to the pulmonary vascular lesions in severe secondary hyperkinetic pulmonary hypertension The clinical symptoms were dominated by cyanosis in the two neonatal subjects In the three cases of older patients, syncopal attacks and/or sudden death was a common feature This indicates that IPH should be considered among the possible causes of sudden, unexpected death in infancy and childhood

The term idiopathic pulmonary hypertension (IPH) implies elevation of the pulmonary arterial pressure primarily due to functional or structural abnormalities of the vasculature of the lung Morphologically, IPH is reflected by hypertrophy of the right cardiac ventricle and hypertensive lesions of the pulmonary vascular bed in the absence of cardiovascular malformations and other possible causes of "secondary" pulmonary hypertension IPH characteristically affects adult females (24), but several cases also in infancy and childhood have been described (1–4, 6–8, 10–12, 14, 18, 19, 21, 24, 25) It has been suggested (1, 8, 11, 12 14, 18, 21, 25) that the pathogenesis of infantile IPH would be different from IPH in adult patients and related to persistance of the foetal type of mural structure in muscular pulmonary arteries

The present report of five autopsy cases of IPH includes two newborn and three older

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currence of aneuploids and chromosome structural aberrations The frequency of polyploids was not altered No effect could be detected with lower cysteine concentrations, or when the cells were treated after the irradiation The protective effect was most clearly manifested by reduction of the number of dicentrics and rings revealed at 48 hours The method described is a contribution, amenable to further development, and to the array of methods used in the study of radioprotective substances

Wennstrom, J, The Folkhalsan Institute of Genetics, Helsingfors CHROMOSOME ABERRATIONS IN MEIOSIS OF MALE MICE ONE DAY AFTER X IRRADIATION

2 month old male mice received whole body irradiation with doses of 50, 100, 200, and 400 rads Meiotic preparations were made one day after treatment and the chromosomes were studied in cells at the diakinesis first metaphase stage of meiosis Chromosome aberrations were 1) acentric fragments, 2) chromatid breaks, 3) trivalent, quadrivalent, pentavalent, and hexavalent like structures, *i e structural aberrations indicating* chromatid exchange

Irradiation was not found to increase the frequency of aneuploid cells The number of fragments per cell increased with the square of the dose This increase is expressed by the equation $y = 2\,8515 \quad 10^{6}D^{2} + 0\,48771 \quad 10^{2}$, in which y = number of fragments per cell and D = dose in rads A similar increase was found for the number of chromatid breaks per cell, the equation in this case being $y = 1\,6500 \quad 10^{6}D^{2} + 0\,5136 \quad 10^{2}$ The number of presumed *chromatid exchanges per* cell increased with the square of the dose, the proportionality being expressed by $y = 0\,746 \quad 10^{6} D^{2} + 0\,2805 \quad 10^{2}$

The results demonstrate that the incidence of chromatid breaks, acentric fragments, and postulated chromatid exchanges in these meiotic cells is proportional to the square of the dose The results *were discussed in relation to the breakage first* hypothesis of Sax (1) and Revell s (2) hypothesis of radiation induced chromatid aberrations

References 1 *Sax, K* Genetics, 25 41 1940—2 *Revell, S H* Mutation Res, 3 34, 1966

Schröder, J, The Folkhalsan Institute of Genetics Helsingfors CYTOGENETIC INVESTIGATIONS OF MEIOSIS OF MICE WITH A SUPPOSED LATENT VIRUS INFECTION

Two mouse strains were investigated as to the condition of their gonads One, the CFW strain (Carmen Farm Webster, virus sensitive) harbours a latent ectromelia infection and the other, the Freiburg strain (originallly a radiation sensitive strain from the Institute of Radiology, University of Freiburg), is apparently free from any kind of viral infection

The CFW strain showed a high percentage of meiotic aneuploidy, and an increased number of *univalency of the sex chromosome pair* Furthermore, a high number of structural chromosomal changes was a characteristic trait of this strain

In the Freiburg strain, on the other hand the frequency of aneoploidy did not exceed that known to be attributable to technical errors The univalency of the X and Y chromosomes was *significantly* lower than that of the CFW strain

The present results suggest that the difference in aneuploidy between the two strains is possibly due to the presence of the ectromelia virus in the CFW *strain* (which is our working hypothesis) while the difference in the univalency of the X and Y chromosomes is probably due to differences between the strains

TABLE 1 *Summary of Clinical Data in Five Cases of Idiopathic Pulmonary Hypertension*

Case	Birth weight (g)	Age at death	Sex	Predominating symptoms	Cardiac catheterization RV pressure (mm Hg)
I	3 310	5 d	♂	cyanosis	–
II	3,210	16 d	♂	cyanosis respiratory distress	102/10
III	3,620	8 mth	♀	sudden death	–
IV	2,680	1 yr 10 mth	♂	syncopal attacks cyanosis sudden death	–
V	3 800	10 yrs	♀	syncopal attacks cyanosis exertional dyspnea sudden death	42/0

unconsciousness and cyanosis, without seizures During the following two years, similar syncopal episodes occurred with increasing frequency, usually at night The duration of the episodes increased from a few minutes to a maximum of 50 minutes She also developed exertional dyspnoea, which gradually became more prominent. Treatment with phenyl ethyl barbiturate did not relieve her symptoms

Cardiac catheterization which was carried out one year after the initial symptoms revealed moderate pulmonary hypertension, with a pulmonary artery pressure of 42/9 mm Hg, and a right ventricular pressure of 42/0 mm Hg

She was readmitted to hospital at the age of 10 years for renewal of the catheterization Roentgenological examination of the chest now showed enlargement of the heart (600 ml/m²), and reduced [illegible]

[illegible] performed

At autopsy the heart showed considerable dilatation and hypertrophy of the right ventricle [illegible] trunk and the aorta near the base of the heart were 75 mm and 68 mm respectively The heart weight was not recorded The lungs were moderately oedematous, without evidence of pneumonia The liver and spleen were congested, but otherwise not remarkable

Both lungs were injected for microangiography

The clinical data of the five cases are summarized in Table 1 Additional features common to all cases were no history of IPH among relatives, and no evidence of abnormal dietary habits in the families of the patients

MICROANGIOGRAPHIC AND HISTOLOGICAL STUDIES OF THE PULMONARY VASCULATURE

METHODS

Microangiography In four cases (*I, II, IV,* and V), the pulmonary arterial system was injected for one hour with 7 5 per cent aqueous suspension of fine barium sulphate (Micropaque®) After fixation in neutral formalin, selected frontal slices of the lungs were embedded in a mixture of paraffin and bees wax From these blocks, 1000 μ thick slices were cut and stereo-microangiographed using a technique described in detail elsewhere (16)

Morphometry The medial thickness of muscular pulmonary arteries was measured in elastin van Gieson stained histological sections from non in-

infants and children Microangiographic and morphometric methods were employed in order to determine whether or not the pulmonary vascular lesions deviate from the pattern usually found in cases of IPH in adult patients

CASE REPORTS

Case I (A 76)

This boy was admitted to the paediatric clinic at the age of 2 days, because of generalized cyanosis and a rough cardiac murmur There was no respiratory distress Roentgenological and electrocardiographic examinations indicated hypertrophy of the right ventricle In spite of treatment with 50–100 per cent oxygen and artificial ventilation, cyanosis persisted The patient died at the age of five days

Autopsy revealed considerable dilatation and hypertrophy of the right cardiac ventricle The heart weighed 35 g (normal 22 ± 4 g) The systemic and pulmonary venous return was not remarkable, and the great arteries were in normal position Dilatation of the pulmonary trunk was recorded, but there was no cardiac malformation The ductus arteriosus was patent to probe The lungs were atelectatic and congested, with aspirated vernix squames in some areas, but there were no inflammatory lesions Both kidneys were dislocated to the pelvis They were reduced in size, with a combined weight of 13 g (normally 27 ± 7 g), but there was no evidence of dysplasia The congested liver showed fatty degeneration of hepatocytes, as well as centrolobular necrosis The spleen too, was the site of marked congestion

The left lung was injected for microangiography

Case II (A 48)

This boy was admitted to the paediatric clinic at the age of 6 days after having suffered from respiratory distress for the last two days The patient had been cyanotic since birth Roentgenological examination of the chest showed considerable enlargement of the heart (580 ml/m²) Cardiac catheterization at the age of 9 days revealed pulmonary hypertension with a right ventricular pressure of 102/10 mm Hg On angiocardiography there was moderate dilatation of the pulmonary trunk The patient died at the age of 16 days

At autopsy, the cardiac enlargement was confirmed The heart weighed 62 5 g (normally 22 ± 4 g) There was dilatation and hypertrophy of both ventricles, of the right one in particular Some minor fenestrations were present in the valve of the foramen ovale, but there was no other cardiovascular malformation The ductus arteriosus was closed The lungs showed moderate atelectasis, and on histological examination there was in addition, some intra alveolar oedema as well as focal intra alveolar and interstitial haemorrhage Irregular oedematous and fibrous thickening of alveolar septa was noted (Fig 2 B), but no hyaline membranes The pulmonary lymphatics were dilated The liver, spleen and kidneys showed moderate congestion, but were otherwise not remarkable

Both lungs were injected for microangiography

Case III (A 139)

This girl had been operated on in the neonatal period for *ectopia ani* and a rectovaginal fistula Following this operation she suffered from recurrent infections of the urinary tract and developed left sided hydronephrosis Otherwise, her postnatal development was not remarkable She died suddenly, during cystoscopy, at the age of 8 months There was no history of previous syncopal attacks

At autopsy, the heart showed no hypertrophy but there was considerable dilatation of the right ventricle The heart weighed 40 g (normally 41 ± 7 g) The gross and histological appearance of the lungs was not remarkable, with the exception of the vascular changes to be described separately below Dilatation of the left ureter and leftsided hydronephrosis were present, and were interpreted as secondary to a stenosis of the distal part of the ureter

Case IV (A 58)

This boy had a normal postnatal development till the age of 1 year and 9 months when he had an attack of unconsciousness and cyanosis without seizures The episode, which had a duration of a few minutes, was followed by spontaneous arousal and vomiting During the subsequent weeks he suffered from multiple similar syncopal attacks and he died suddenly at home one month later

Autopsy revealed cardiac enlargement with dilatation and prominent hypertrophy of the right ventricle The heart weighed 84 g (normally 60 ± 11 g) There was no cardiovascular malformation but the pulmonary trunk was somewhat wider than the aorta The circumferences of these vessels near the base of the heart were 61 mm and 49 mm respectively The gross and microscopic appearance of the pulmonary parenchyma was normal except for the vascular lesions (see below) The liver and spleen were congested but otherwise not remarkable

Both lungs were injected for microangiography

Case V (A 112)

This girl had a normal postnatal development till the age of 8 years, when she had an attack of



Fig 2 Histological features of the lungs in two neonatal cases of IPH

A Thick walled muscular pulmonary arteries (*arrows*) in case I, age 5 days Elastin van Gieson × 190

B Oedematous and fibrous thickening of alveolar walls in case II age 16 days Two unduly thick walled small muscular pulmonary arteries are indicated by *arrows* Haematoxylin and eosin × 190

jected lung specimens available in two cases (I and III) The media index was calculated according to the principles outlined by Wagenvoort (22), as the mean ratio between medial thickness and external diameter in cross sectioned muscular pulmonary arteries

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Fig 1 Intralobular arterial pattern in normal perinatal lung and in two neonatal cases of IPH

A Normal perinatal intralobular arterial pattern in stillborn infant (BW 2670 g) without cardiovascular abnormalities Microangiogram × 12

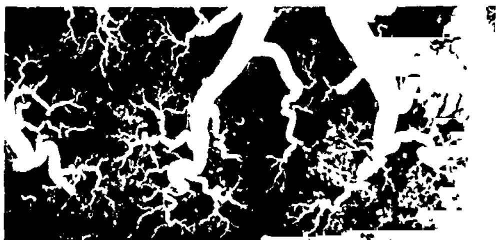
B Pulmonary microangiogram from Case I age 5 days showing some abnormally tortuous intralobular arteries × 12

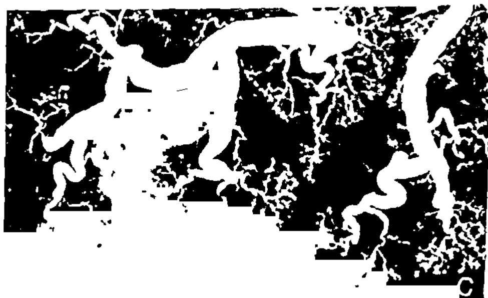
C Prominent tortuosity of intralobular pulmonary arteries in case II age 16 days Microangiogram × 12

RESULTS

Pulmonary microangiograms from the two neonatal cases (I and II) revealed abnormal tortuosity of many intralobular pulmonary arteries (Fig 1 B and C) This feature was particularly prominent in Case II Otherwise, the microangiographic pattern of these two cases was not remarkable, without evidence of abnormal arterial bronchopulmonary anastomoses On histological examination, the muscular pulmonary arteries and arterioles in both cases showed persistence of foetal structure and irregular medial hypertrophy (Fig 2 A and B) The latter feature is also reflected by the high media index (0 22) which was obtained on morphometry of the muscular pulmonary arteries in case I


A


B


C

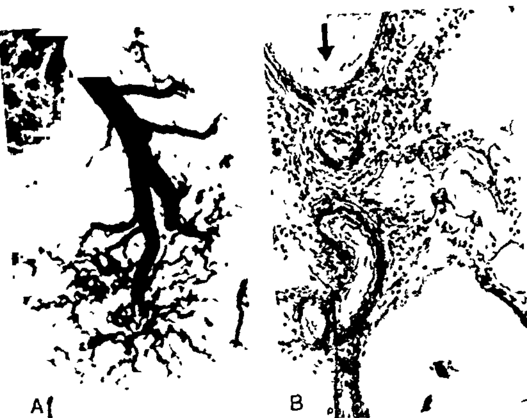


Fig 4 Caput Medusae like intralobular arterial pattern due to obliterative intimal fibrosis in muscular pulmonary artery and filling of the capillary bed of the lobulus peripheral to the obliterated arterial segment by means of intralobular collaterals Obliterated arterial segment is indicated by corresponding *arrows* in A and B Case V, age 10 years

A Microangiogram × 16

B Selected serial section showing obliterated muscular pulmonary artery (*arrow*) and contrast-filled, dilated pre-capillaries (*centre*) Elastin van Gieson × 180

were found in case V Both cases showed focal mural fibrinoid necrosis of occasional, small, muscular pulmonary arteries, without concomitant inflammatory reaction

The microscopic appearance of the pulmonary veins was normal in all cases

COMMENT

The reliable diagnosis of IPH should be based upon the clinical verification of the hypertensive state, preferably by means of cardiac catheterization, and on the exclusion of cardiovascular malformations and other disorders capable of causing secondary pulmonary hypertension Cardiac catheterization was performed in only two cases in the present series, in the remaining three cases a diagnosis of pulmonary hypertension was obtained [illegible] diac ventricle at autopsy, and hypertensive lesions of the pulmonary vasculature

Cardiovascular and pulmonary disorders known to cause secondary pulmonary hypertension were excluded at autopsy in all cases However, the pulmonary lesions in case II



Fig 3 Lung section from case III age 8 months showing prominent medial hypertrophy in transitional pulmonary arteries (*arrows*) Elastin van Gieson × 210

In case III, microangiographic examination was not performed The histological appearance of the pulmonary arterial system was characterized by prominent medial hypertrophy, affecting muscular and transitional pulmonary arteries (Fig 3) In this case, the media index was the same as that obtained in case I *i e* 0 22

Cellular intimal proliferation and intimal fibrosis were absent in these first three cases of the series Thrombotic or plexiform lesions were not found nor was there evidence of necrotizing arteritis

The microangiographic pattern in cases IV and V was different from that in the aforementioned three cases and characterized by a very irregular capillary filling and focal 'pruning' of the intralobular pulmonary arteries Tortuosity of the most peripheral pulmonary arteries and overdistension of many precapillary vessels yielded a bushy in places caput Medusae like, intralobular arterial pattern (Fig 4 A)

In case IV, there was a retrograde contrast filling of numerous pleural systemic arteries particularly at the dorso mediastinal aspect of the right upper lobe These pleural arteries were very tortuous and ramified into the underlying pulmonary parenchyma (Fig 5A) In this case the microangiograms also revealed several abnormal pulmobronchial arteries with prominent terminal ramifications to the alveolar capillary bed (Fig 5 B)

On histological examination a varying degree of medial hypertrophy in muscular pulmonary arteries was observed both in case IV and case V These arteries also showed focal cellular intimal proliferation and obliterative intimal fibrosis (Fig 4 B)

Peripherally to obliterated segments of the muscular pulmonary arteries dilated contrast filled precapillaries and capillaries were encountered in some areas In case V the contrast seemed to have reached these vessels largely by means of intralobular pulmonary artery derived collaterals which contributed to the above mentioned irregular bushy microangiographic pattern (Fig 4)

Numerous plexiform lesions were demonstrated in case IV (Fig 6) but no such lesions



Fig 6 Microangiographic and histological appearance of a plexiform lesion indicated by corresponding *black arrows* in A and B Case IV age 1 year and 10 months.

A The plexiform lesion stands out microangiographically as a tortuous narrow portion of a muscular pulmonary artery proximal to a dilatation lesion (*white arrow*) Microangiogram × 58

B Selected serial histological section from the same area showing the plexiform and dilatation lesions Elastin van Gieson × 92

means of the foramen ovale and the ductus arteriosus Provided that these pathways are not prematurely closed structural or functional abnormalities of the foetal pulmonary vasculature would seem to have little chance to overload the right ventricle

Involution of the media of muscular pulmonary arteries is a characteristic feature of the normal postnatal development of the lung Due to this involution the media index of muscular pulmonary arteries drops from a level of 15–20 per cent at term to about 10 per cent at the age of one month (23) In neonatal cases of hyperkinetic pulmonary hypertension secondary to *e g* ventricular septal defect the normal postnatal involution of the media of muscular pulmonary arteries does not take place Instead there is carry over of the foetal structure of these arteries which constitutes the initial stage of the hypertensive vascular lesions (9) In *the two* neonatal cases in the present series (I and II), a similar persistence of the foetal structure of muscular pulmonary arteries was observed, with moderate superimposed medial hypertrophy

Also *in case III there* was medial hypertrophy in muscular pulmonary arteries However, in this case it cannot be determined whether this was the result of persistence of

deserve a special comment. This infant had suffered from respiratory distress, manifest since the age of 4 days. Atelectasis without hyaline membranes was observed in the lung sections, as well as interstitial oedema and irregular fibrosis of alveolar walls. The aetiology of these pulmonary lesions is uncertain, but the histological appearance is compatible with a late stage of hyaline membrane disease, *possibly modified by oxygen treatment* (cf. 15). *In this case, it cannot be excluded* that the lesions of the pulmonary parenchyma might have contributed to the pulmonary hypertension. It is well known that hypoxia causes arterial vasoconstriction in the lungs, and hence an increase of the pulmonary vascular resistance, but so far there is no evidence suggesting that persistent pulmonary hypertension can be initiated by neonatal respiratory distress.

Cases I and II are similar to the few neonatal cases of "primary pulmonary arteriosclerosis" previously reported (25). The *pathogenesis of isolated right ventricular hypertrophy in the immediate neonatal period* is, however, less obvious than in cases of IPH in older patients (cf. 13). In the foetal circulation, the lungs are largely by passed by

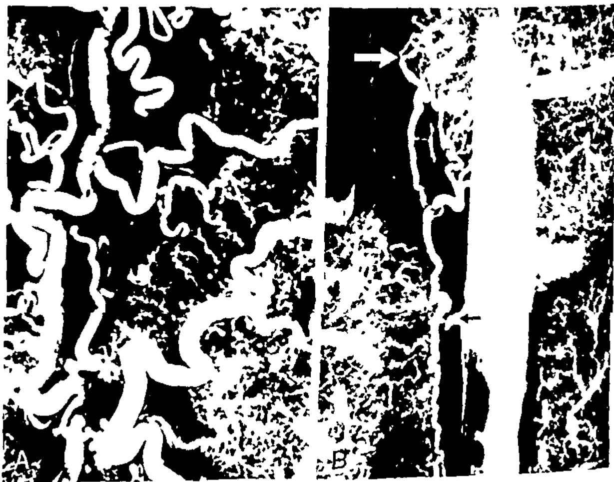


Fig. 5. Collateral arterial pathways in the lungs of case IV, age 1 year and 10 months.

A. Pleural systemic arteries in the right upper lobe. The arteries are tortuous and ramify into the underlying pulmonary parenchyma. The course of these vessels has been verified by serial sectioning. Microangiogram × 11.

B. Pulmobronchial artery (diameter 200 μ), the origin of which is indicated by *black arrow*, with concurrent and recurrent branches in the bronchial wall and with unusually prominent terminal ramifications to the alveolar capillary bed (*white arrow*). The course of the pulmobronchial artery has been verified by serial sectioning. Microangiogram × 11.

would expect that if the parathyroids had not developed adequately the thymus, in many instances at least, might also be hypoplastic

Therefore, re investigation of the possible role of the parathyroids as a cause of sudden death in infancy was instituted

MATERIAL

These data are derived from a total of 137 post mortem examinations conducted between February 1967 and June 1968 Of these *54* were from The Office of the Medical Exam ner of the State of Maryland in Baltimore *3?* from St Christopher s

[illegible]

Columbia

Eighty eight of the infants had died suddenly and unexpectedly and their deaths remained unexplained following complete necropsy with examination of the brain and of adequate numbers of histologic sections The other 49 infants died of recognized ca [illegible] 3 [illegible] pn [illegible] dia [illegible] (a and vomiting) 20 of major congenital anomalies (10 *of* whom had severe *congenital* heart disease) 10 of trauma suffocation burns or poisoning and 5 of miscellaneous causes

The test (88) and control (49) groups were comparable with respect to sex with a preponderance of males in each All infants were less than one year of age There were however relatively more Negro children among those dying suddenly and unexpectedly than among the controls (64 per cent as opposed to 54 per cent)

METHOD

At autopsy the organs of the neck were removed in block This included the tongue superiorly and extended inferiorly to the level of the clavicles laterally the block of *tissue passed to the* internal carotid artery on each side and antero posteriorly from the skeletal muscle or subcutaneous tissue to the ventral surfaces of the vertebral bodies This block of *fresh tissue* was refrigerated until it was dissected usually a day or so after completion of the gross autopsy

Dissection was accomplished with a stereoscopic microscope using a magnification of 4 times The block of tissue was oriented so that the posterior wall of the esophagus was directed toward the lens Each nodule thought to be parathyroid was delicately lifted away *from* the block, all thymic nodules adherent to parathyroids were taken in continuity with them

Each structure removed from the block was labeled separately and its location plotted in a drawing Subsequently, following histologic confirmation of their identity the position of each parathyroid and thymic nodule was affirmed in the original diagram

In only eleven of the infants were fewer than two parathyroid glands identified grossly, in each of these modified serial sections of the organs of the neck were prepared and every fifth section each 5 microns in thickness was examined

Far greater difficulty was encountered in locating the lower parathyroids than the *upper ones* (Table 3) This can be attributed to two factors (1) The position of the upper glands is far more [illegible] tion of the lower glands, on the other hand, is extremely variable, they may lie against the lower pole of *the thyroid posteriorly*, medially or inferiorly or be located at quite a distance from the thyroid, usually caudal to it (2) Although any parathyroid may be hidden beneath the capsule of a thymic nodule and thus obscured from view this is much more apt to occur with the lower glands than the upper (Table 3)

RESULTS

At least two parathyroid glands were identified in all of the 88 infants who had died suddenly and in all but two of the controls, in the latter group one gland was found in one infant and none in the other The average number demonstrated per case was 2 9 for the crib deaths and 2 8 for the controls (*Table 1*) There was no substantial discrepancy between the two groups of infants with regard to the locations of the glands identified (Table 3)

In many instances, parathyroid and cervical thymic tissue were closely approximated, the thymic tissue under *these circumstances* was sometimes continuous with the main body of the gland, and at other times it was entirely independent of it *In 41 per* cent of the infants who had died suddenly and in 26 per cent of the controls the two types of tissue were separated from each other by a thin fibrous septum. Intimate fusion of the

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THE PARATHYROIDS IN SUDDEN, UNEXPECTED DEATH IN INFANTS*

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Two or more parathyroid glands, of normal gross and histologic appearance, were identified in all of 88 infants who had died suddenly and unexpectedly and in 47 of 49 controls of similar age Close approximation or intimate fusion of the parathyroid with cervical thymic tissue, either a cervical continuation of the major thymic mass or, more frequently, an independent nodule of thymic tissue, was observed in 58 per cent of the infants who had died suddenly and in only 37 per cent of the controls Such union is considered to be a variant of normal in the infant In many of the controls the stress of the terminal illness not experienced by the infants who had died instantaneously, may have been responsible for involution of such cervical thymic masses so that they were no longer readily demonstrated

In 1965, *Geertinger* (2) first proposed the hypothesis that sudden unexpected death in infancy is usually the result of inadequacy of the parathyroids He reported total absence of any parathyroid tissue in 34 per cent of 82 such infants and union of parathyroid with cervical thymic tissue in another 32 per cent, this close contact or intimate association of the two types of tissue he considered to be morphologic evidence of "incomplete fetal development" and, by implication, of inadequate function Thus, he concluded that some abnormality of the parathyroids was present in 66 per cent of the sudden deaths in his series and that these deficiencies had "lead to a disturbance of the calcium regulating mechanism and thence to a sudden hypocalcemic catastrophe (triggered by mild infections and other relatively trivial causes)

This theory seems unlikely for two reasons (1) Clinically, the infant who dies suddenly and unexpectedly has usually been relatively well prior to death, by definition, there is no history of serious illness or repeated convulsions such as might be expected with parathyroid insufficiency, (2) in infants who die a so-called "crib death" the thymus is relatively large, well preserved and plump Since the thymus and parathyroids are derived from the same embryologic anlagen one

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* Preliminary reports of this study were presented before the Pediatric Pathology Club in October, 1967 and the Second International Conference on Sudden Death in Infants in February, 1969 (1)

sible for any of these deaths The same con clusion was reached by *Ellis* & Knight (4) who also *found* at least two parathyroid glands in each of 31 infants dying suddenly and unexpectedly

Dr *Geertinger's* inability to demonstrate any parathyroid tissue *in* 34 per cent of the infants in his series may be attributed to the method employed The thyroid was removed dissected free of the trachea and surround ing tissues and then "cut into small pieces which were sectioned serially (3) Parathyroid glands located at almost any distance from the thyroid may have been lost in the first maneuver Moreover the second procedure precluded localization of those parathyroids which were found What is not so easily understood however is the apparent absence of parathyroid tissue in only 7 per cent of the controls in whom this same tech nique was utilized

Although *Geertinger* states that anatomical union or approximation of parathyroid with thymic tissue reflects "congenital incomplete development" of the parathyroids and im plies that a parathyroid so situated would not function adequately (3), there is no evi dence for either of these assumptions

Particularly intriguing in our own experi ence however is the *difference between the* two groups of infants with regard to such approximation Union of one type or the other was observed much more frequently among *infants who had died suddenly* (58 per cent) than among controls (37 per cent) ($P \approx 017$) (Table 2) This may be due to involution of thymic tissue under conditions of stress *The majority of the control infants* in this *study had* been ill prior to death and thus *relatively* small depleted masses of thymic tissue *might* not have been identified By *contrast such involution* could not have occurred among the test infants whose deaths had been virtually instantaneous thymic nodules would be expected to be *well main* tained and *easily visible*

Further to support his hypothesis regarding parathyroid insufficiency *Geertinger* has re ported lower levels of serum calcium in test infants than in controls (3) In a separate investigation we have determined the levels of serum calcium in 75 infants, 57 of whom had died suddenly and unexpectedly and 18 of whom served as controls, all of the autop sies were performed less than 12 hours after death The level of serum calcium was less than 7 mg per cent in only two infants in each group Thus it would seem that hypo calcemia, like hypoparathyroidism, *cannot* be *incriminated as a cause of "crib deaths*

CONCLUSION

Two or more parathyroid glands of normal gross and histologic appearance have been demonstrated in 88 infants who died sud denly and unexpectedly It is concluded that parathyroid insufficiency is not responsible for sudden death in infancy

Union or approximation of parathyroid *with* cervical thymic tissue in the infant is probably a variant of normal and there is no reason to believe that it has any bearing on the function of either gland Our observation *of it in more of the infants who had died* suddenly (58 per cent) than in controls (37 per cent) is attributed to involution and consequent apparent disappearance of thy *mic tissue during the* stress of terminal illness in the latter group

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TABLE 1 *Demonstration of Parathyroid Glands*

	Sudden deaths	Controls	Total
Total number of infants	88	49	137
Number of infants in whom 2 or more parathyroid glands were demonstrated	88	47	135
Average number of parathyroid glands demonstrated per infant	2 95	2 79	2 89

TABLE 2 *Union of Parathyroid Glands with Thymic Tissue*

	Sudden deaths	Controls	Total
Total number of infants	88	49	137
Number of infants with abutment of thymic and parathyroid tissue with a separating fibrous septum	36 (41%)	13 (26 5%)	49 (35 7%)
Number of infants with fusion of thymic and parathyroid tissue without any separating fibrous septum	19 (21 6%)	5 (10 2%)	24 (17 5%)
Number of infants with either abutment or fusion of thymic and parathyroid tissue	51*(58%)	18 (36 7%)	69 (50.5%)

* Four of the infants who had died suddenly exhibited abutment involving one gland and fusion involving another

TABLE 3 *Position of the Parathyroid Glands Identified*

	Total* number of infants in the study	Number of parathyroid glands identified as to position: Right, Upper, Total	Right, Upper, Junction§ with thymic tissue	Right, Lower, Total	Right, Lower, Junction with thymic tissue	Left, Upper, Total	Left, Upper, Junction with thymic tissue	Left, Lower, Total	Left, Lower, Junction with thymic tissue
Sudden deaths	88	78	6	53	24	79	4	49	29
Controls	48	36	2	27	12	42	0	28	8

* Does not include one control infant in whom the specific locations of the parathyroid glands were not determined

§ Includes both "abutment" and 'fusion' of parathyroid with thymic tissue

two without any intervening septum and with admixture of their cells at the line of approximation was present in 22 per cent of the sudden deaths and in only 10 per cent of the controls (Table 2) These correspond to the "incomplete" and "complete" fusions, respectively, described by *Geertinger* (3)

DISCUSSION

Since two or more parathyroid glands of normal gross and histologic appearance were identified in all of the 88 infants who died suddenly and unexpectedly, it is concluded that parathyroid inadequacy was not respon-

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LIMITATIONS OF CHROMOSOME ANALYSIS AS A METHOD FOR BIOLOGICAL DOSIMETRY OF CHRONIC EXTERNAL RADIATION AND OF INTERNAL EXPOSURE TO THERAPEUTIC RADIOPHOSPHORUS

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A comparison between the results of chromosome analyses from persons exposed to chronic external irradiation and chromosome analyses from patients having received treatment with radiophosphorus shows that chromosome aberrations in the peripheral blood are found in both cases and that the aberrations are similar. A quantitative correlation between the radiation dose from chronic external exposure and the aberration yield will involve so many unknown parameters that it seems irrelevant. A quantitative determination of the chromosome aberrations can however, when sufficient data have been collected, probably be used for biological dosimetry, provided that the exposure is approximately uniform. A correlation between the amount of radiophosphorus administered and the aberration yield cannot be used for biological dosimetry at least if the treatment, as is the case in practice, is given discontinuously.

In the Report of the United Nations Scientific Committee on the Effects of Atomic Radiation 1969 (8) a large chapter is devoted to radiation-induced chromosome aberrations in human cells. A central question is the relationship between aberration yield and radiation dose, a subject with which a number of laboratories has been occupied for more than five years. One of the reasons why this has been the subject of such intensive research is among other things the possibility of using the chromosome analyses for biological dosimetry. Work has mainly been done with in vitro systems, i.e. irradiated blood cultures, but the various laboratories have obtained diverging results on the relationship between aberration yield and radiation dose. Investigations of these differences have also been commented in the U.N. report.

Further it has been discussed whether it is possible to correlate the results from in vitro experiments with results after in vivo exposure. For human material a final answer has not been obtained so far as opportunities for

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ADDENDUM

After this paper was submitted for publication we became aware of another article by Dr Geertinger (Parathyroid Abnormalities a histological study with special reference to sudden, unexpected death in infancy Acta Pathologica et Microbiologica Scandinavica 75 270–272, 1969) Serial sections of the organs of the neck were examined in 17 instances of sudden death in infancy There were *no* examples of absence of the parathyroid glands, the average number of glands identified per patient being 2 7, a figure comparable to that observed by us (2 9)

Dr Geertinger noted fusion of parathyroid and thymic tissue in 21 of the 30 lower parathyroids, *i e*, 70 per cent, this is even higher than observed by us in our sudden deaths, *i e*, 52 per cent We would agree with him that such thymic parathyroid fusion probably is a common variant of normal

Since the evidence would indicate that only insignificant amounts of calcitonin (if any) are produced by the parathyroids, his implication that absence of the upper glands would be associated with significantly diminished amounts of calcitonin and by that means related to sudden death in infancy would appear to be fallacious

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TABLE 1 *Dose Measurements and Chromosome Aberrations in Peripheral Blood Cultures from Personnel Chronically Exposed to External Radiation*

Age (yrs) sex	Period of measurement	Dose measured (R)	Employed before start of measurement	Total cells analysed	% cells with unstable aberrations	% cells with stable aberrations
27 ♀	Non irradiated controls			50	2	0
24 ♀				50	2	0
32 ♂				50	0	0
42 ♀				50	0	0
41 ♀				50	2	0
35 ♀				50	2	2
35 ♂	4 mths	1 240	–	100	0	0
51 ♀	2 mths	17 240	–	100	1	0
42 ♀	7 yrs	19 100	–	50	0	0
46 ♀	2 yrs 7 mths	27 695	–	100	1	0
44 ♀	7 yrs 6 mths	>63 355	–	100	4	1
43 ♀	10 yrs 4 mths	67 375	2 yrs 4 mths	100	7	0
44 ♀	10 yrs 3 mths	74 330	1 yrs 5 mths	100	2	1
57 ♀	10 yrs 3 mths	>77 225	13 yrs 9 mths	100	4	1
59 ♀	10 yrs 4 mths	>93 180	4 yrs 7 mths	100	5	1
50 ♀	10 yrs 4 mths	>116 110	3 yrs	100	2	2

chromosome analyses are made at a laboratory with a standardized technique The exact curve for the decrease of cells with chromosome aberrations in relation to time must be known This curve varies greatly among individuals and probably also among different age groups A measure of the qualitative and quantitative variations of the lymphocyte population is also essential It would be necessary to know the quality of radiation and dose rate and the variations of order of magnitude of the exposure In other words, an attempt to obtain an *in vivo* correlation between radiation dose and aberration yield in persons exposed to chronic external radiation is for the time being hardly practicable

It is, however, evident that as in the case of acute accidental exposure the chromosome aberration yield in the occupationally exposed staff reflects the immediate biological condition If the exposure was approximately uniform, it would be possible to imagine such chromosome analyses, evaluated on the basis of average values collected, used in a kind of biological dosimetry for the purpose of forming a basis for maximum permissible dose, supplementing a physical measurement, as these analyses reflect purely biological conditions For statistical reasons this would necessitate analysis of a very great number of cells, and owing to a number of the mentioned uncertain parameters in the chromosome analyses the aberration level could, however, only form the basis of a rough estimate

16*

B CHROMOSOME ABERRATIONS IN PATIENTS TREATED WITH ^{32}P FOR POLYCYTHAEMIA VERA

In the field of medicine therapeutic isotope treatment is still used, though in a greatly diminishing degree It must be considered as a kind of chronic internal exposure to radiation Among the isotopes that are still used in fairly large doses is ^{32}P which is mainly used in the treatment of patients with polycythaemia vera ^{32}P is an effective therapeutic agent with a number of advantages compared with other methods of treatment of this disease The isotope treatment is, how-

examining individuals exposed to large radiation doses have fortunately been very few The available, scant data do, however, indicate a certain agreement between dose-response relationship *in vitro* and *in vivo* (2, 3)

The evaluation of the results of the chromosome analyses does, however, as mentioned below, involve a great number of more or less unknown parameters The greater part of the experiments carried out until now and the *in vivo* examinations have been concerned with single exposures The possibility of using chromosome analyses for determination of dose is considerably more complicated where continuous or fractionated exposure is concerned, and also in these cases the *in vivo* conditions are particularly difficult to evaluate Only a small number of studies have been made of persons exposed to chronic radiation, comprising individuals who have received chronic low doses from external scources and patients treated with isotopes of various kinds

The present study comprised two such groups, namely the occupationally exposed staff of the gynaecologic unit at the Radium Centre, Copenhagen, and a group of patients from "Radiumhemmet", Stockholm, who for years have been treated for polycythaemia vera with radiophosphorus The purpose of presenting this material is not so much that of showing the results, but should rather be regarded as a contribution to the discussion of the limitations of the value of these analyses as a method for biological dosimetry

A CHROMOSOME ABERRATIONS IN CASES OF CHRONIC EXTERNAL EXPOSURE TO RADIATION

The main purpose of the examination of the staff exposed to radiation was the clarification of the extent and the types of chromosome aberrations in the peripheral blood of these persons, and the evaluation and discussion of the importance of the chromosome aberrations, and particularly the value of the chromosome analyses for biological dosimetry

MATERIAL AND METHODS

The material comprised ten persons, all between 35 and 59 years of age, who had been employed at the Radium Centre, where measurements of the exposure to radiation had been made for a number of years with 'Cintel's C K Dosimeter' As will be seen from Table 1, measurements have been performed for as long as ten years Several persons have, however, been employed for a period longer than this, and for these persons the measured total dose is consequently too low Besides, in a few cases, the dosimeter had reached its maximum at the time of changing, and the dose stated is therefore too low This is denoted by > in the Table

RESULTS

The results of the examination of the staff exposed to radiation have been published elsewhere (11), but they are included here as a basis for discussion of biological dosimetry and in order that parallels may be drawn from chromosome analyses after chronic external exposure to the results of chromosome analyses made on ^{32}P-treated patients

It appears that a small percentage of cells with chromosome aberrations, mainly "unstable", has been demonstrated even in persons exposed to less than the maximum permissible dose There is a statistically significant difference at the 5 per cent level between the 6 persons having received the most heavy irradiation and the controls, but otherwise, no definite relationship between measured dose and aberration yield was found Similar results have been obtained by a number of other investigators, among them *Bauchinger & Hug* (1) who have made a study fairly similar to the present

DISCUSSION

The drawing up of a relationship for practical use between radiation dose and aberration yield for persons occupationally exposed to radiation is very complicated, even when the

TABLE 2 *Chromosome Aberrations in ³²P Treated Patients with Primary Polycythaemia*

Age (yrs) sex	Period of treatment	Dose ³²P mCi	Peripheral blood			Bone marrow		
			Total cells analysed	% cells with unstable aberrations	% cells with stable aberrations () % clone	Total cells analysed	% cells with unstable aberrations	% cells with stable aberrations () % clone
74 ♀	Untreated		–	–	–	50	0	0
71 ♂	Untreated		–	–	–	30	0	0
24 ♂	Untreated		50	0	0	50	0	0
46 ♂	Untreated		–	–	–	32	0	9
78 ♀	1961 69	67	20	25	15	100	0	0
53 ♂	1959 68 (1966 1968 Myleran)	60	–	–	–	34	0	0
58 ♂	1958 68	54	23	9	13	75	0	0 (35)
80 ♂	1961 68	54	20	15	0	51	0	63 (57)
83 ♀	1962 69	42	–	–	–	23	0	87 (83)
81 ♀	19 ? 58 1958 69	? 40	–	–	–	51	0	38 (32)
72 ♂	1956 68	38	38	13	18	12	0	0
64 ♀	1955 67	35	100	8	11 (4)	49	0	94 (94)
78 ♀	1961 67	35	78	4	9 (1)	12	0	75 (58)
62 ♂	1963 68	35	32	0	0	60	0	73 (73)
73 ♂	1957 67	34	14	0	0	100	0	58 (58)
69 ♀	1950 68	30	13	(3 cells)	(3 cells)	21	0	0
60 ♂	1961 67	30	–	–	–	53	0	87 (87)
83 ♀	1959 68	20	–	–	–	18	0	0
76 ♀	1956 66	19	8	0	0	50	0	2
64 ♀	1963 64	16	50	2	0	26	0	73 (61)
65 ♂	1967 (1965 68 Myleran)	14	77	14	5	50	0	6
73 ♀	1940 68 (1968 wholebody irr)	?	–	–	–	100	0	91 (91)
72 ♂	(1966 spleen irr) (1966 67 Myleran		45	15	4 (2)	79	0	100 (100)
54 ♂	(1965 69 Myleran)		–	–	–	50	0	0
51 ♂	(1968 69 Myleran)		6	0	0	13	0	0

ever, now being limited as much as possible (9) owing to the considerable radiation exposure of the patients and the involved possibility of an increased incidence of leukaemia. At "Radiumhemmet" in Stockholm there is a fairly large, uniform material of ^{32}P-treated patients, and in a collaboration between Stockholm and Copenhagen chromosome analyses have now for the past almost two years been made on blood and bone marrow from a group of patients with polycythaemia vera, mainly those who through the years have received rather large isotope doses. The investigations have many theoretical and practical aspects, but here we shall not deal with the medical circumstances, but concentrate on the purely radiation biological aspects of the dose-response relationship.

MATERIAL AND METHODS

The material comprises 25 patients (Table 2). Of the 21 treated patients 9 were without myelofibrosis, 5 had myelofibrosis, and in 7 cases early transition into leukaemic phase could not be excluded.

RESULTS AND DISCUSSION

Peripheral Blood

As mentioned examinations have been made of peripheral blood as well as of bone marrow. A number of circumstances often makes it technically difficult to produce satisfactory preparations from peripheral blood for chromosome analyses from these patients (5), with our material we failed in approximately forty per cent of the cases.

Concerning the results of examinations of the peripheral blood, it appears that the types of aberrations that are found correspond to what was found following chronic external exposures. First and foremost there is a number of "unstable" and "stable" forms of aberrations in the treated patients, and the karyotypes are balanced. There was no definite relationship between the total quantity of ^{32}P administered and the chromosome aberration yield found in the peripheral blood in the material. Nor was this to be expected for the same reasons as stated in the case of chronic, external exposure. To this should be added other individual circumstances such as distribution of ^{32}P, vascularization, etc. Finally, the uncertainty as to the actual exposure to radiation from the ^{32}P administered must be borne in mind. It has been estimated that 1 mCi injected ^{32}P corresponds to 51 rems to bones (10), but recent reports indicate that 1 mCi ^{32}P is more likely to equal 85 rads (6). However, as mentioned for the chronic, external exposure, conditions may again be evaluated from the point of view that the level of chromosome aberrations may reflect the immediate situation in the patients, hypothetically one might imagine a continuous isotope dosage, and on the basis of experience concerning average values it should then be theoretically possible to reach a relationship between the quantity of ^{32}P administered and the aberration yield. However, the evaluation of the aberration yield would again be subject to great uncertainty.

Bone Marrow

In the chromosome analyses the examination of the bone marrow gave results quite different from those for the peripheral blood. The types of chromosome aberrations found were mainly "stable" aberrations, but in twelve of the treated patients formation of clones with specific chromosome patterns were also detected. These clones must be considered as originating from one cell, and the question arises whether this is a single radiation damaged cell, or whether the cell has connection with the disease of the patient. This question has not been solved. As first pointed out by *Kay et al.* (4) there may be a connection between a deleted F chromosome and the disease. The question whether the karyotypes of the clone cells are balanced in analogy with findings in most radiation experiments, i.e. whether the karyotypes contain the normal amount of chromatin has been evaluated. This problem has not been solved so far, but progress may be possible in the near future by means of an ultra

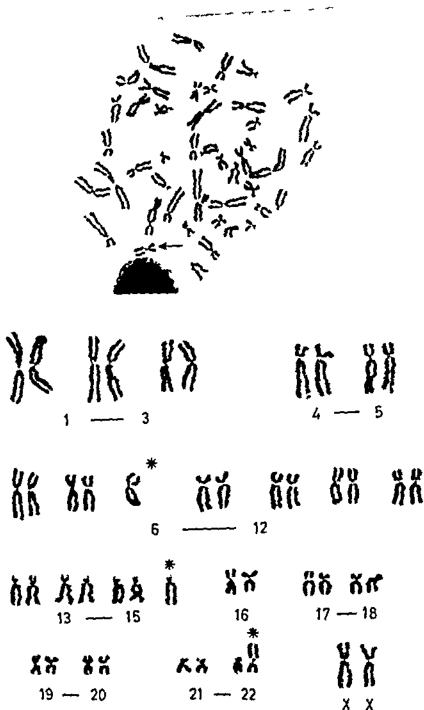
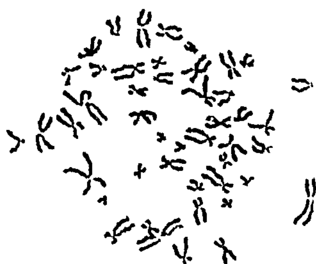
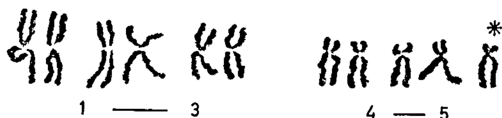
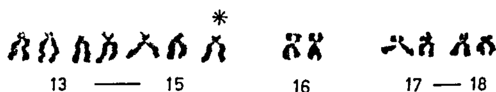


Fig 2 Karyotype of clone cell from ^{32}P treated patient with primary polycythaemia Translocation of material from a chromosome in the C-group to a chromosome in the G-group Presumably balanced karyotype









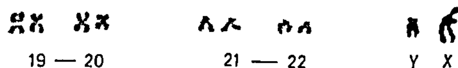


Fig 1 Karyotype of clone cell from ^{32}P-treated patient with primary polycythaemia Translocation of material between two chromosomes in the C group Presumably balanced karyotype

microfluorometric technique If the karyotypes are balanced it will in our opinion support the theory that these cells are primarily radiation damaged cells As mentioned above, the clones were detected almost exclusively in the bone marrow samples, only in rare cases a few clone cells were demonstrated in the peripheral blood Till now the clone cells have been found in treated patients only, but it must be added that only few untreated patients have been examined By far the greater number of the clones probably have balanced karyotypes, but at least in one case the karyotype could not be balanced as the clone cells in this patient contained 47 chromosomes with an additional chromosome in the C-group

The clones comprised from 32 to 100 per cent of the analysed cells from the bone marrow A comparison between the presence of the clones and the ^{32}P dose administered does not show any definite relationship as there is a fairly large variation in the quantity of isotopes given to the patients presenting the clones —Thus, neither the presence of the clones nor their size can be used for a dose response relationship This was not to be expected if the theory claiming that the clones originate from a single, radiation-damaged cell proves true The presence of the clones is, however, of great clinico theoretical interest as it must be considered whether or not the clones represent malignant cell populations It should be realized that the clones are often present to an extent suggesting that these cells have selective advantages Still this need not mean they are malignant, but some of our patients in whom clones were found later died presenting a picture of acute or subacute leukaemia (12, 13) We have also had the opportunity to follow a clone formation that increased as the patient s condition deteriorated, until shortly before death 100 per cent cells were found with the same specific chromosome pattern

At the same time it must however be stressed that with the majority of the patients with the clones there is not the least suspicion clinically of leukaemia or of a transition into leukaemic phase As a working hypothesis it may be assumed that the clone cells represent populations primarily originating from radiation damaged cells that show an increased tendency to transition into malignancy, and that in certain cases we see the clone before the transition has taken place, in others after

It is known that there are a number of conditions in which patients with specific chromosome abnormalities have an increased tendency to leukaemia Best known in this connection are diseases such as Down's syndrome, Klinefelter's syndrome, Fanconi's anaemia, Bloom s syndrome, and ataxia-teleangiectasia One may assume that the chromosome aberrations in these diseases increase the "sensitivity" of the cells to neoplastic agents, and experimental work with SV 40 virus seems to support this theory (7)

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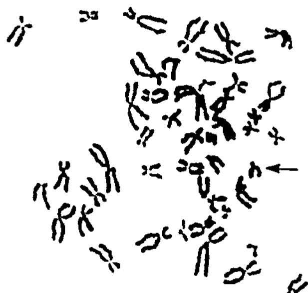
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X X

Fig 3 Karyotype of clone cell from ^{3} P treated patient with primary polycythaemia Translocation of material from a chromosome in the D group to a chromosome in the E group Presumably balanced karyotype

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HISTOLOGICAL CHANGES IN LONG TERM EXPLANTS OF HUMAN LYMPH NODES DURING LYMPHOBLASTOID TRANSFORMATION

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The sequence of events in long term cultures in lymph node fragments and in underlying three dimensional matrix of gelatin foam (Spongostan) has been studied histologically Lymphoid cells together with reticulum like cells survived excellently in the periphery of the original lymph node fragments until the time of lymphoblastoid transformation 8 weeks after explantation The centre of the lymph node became increasingly fibroid from the third week onwards The Spongostan matrix was gradually converted to an organotypic structure where lymphoid cells survived and proliferated During week 3–6 after explantation only few lymphoid cells were discharged into the medium At the same time, however, the number of such cells did not decline within the explant The diminished release could thus not be explained by increased lymphoid cell death at their source of origin, *i e* the original explant The findings support that lymphoblastoid cells go through a phase of adaptation within the explant

During the last few years a large number of permanent lymphoblastoid cell lines have been established *in vitro* The majority of these has come from patients with lymphoma /leukaemia or mononucleosis but lines have also been derived from apparently healthy individuals as reviewed by *Ponten* (1971)

The first lymphoblastoid lines were obtained in low frequency from bone marrow biopsies from children with leukaemia or acute infectious mononucleosis (*Benyesh Melnick et al* 1963) It was suggested that the lymphoblasts in these cultures originated from bone marrow fibroblasts transformed *in vitro* The term lymphoblastoid transformation was used to describe this phenomenon

Many different techniques have been employed and the frequency of lymphoblastoid transformation has varied within wide limits A modified Trowell-Jensen grid organ culture where lymph node fragments are placed within a piece of wetted gelatin foam has given the highest frequency (almost 100 per cent) of established lines (*Nilsson* 1971) In previous reports where grid organ cultures were employed, only the cells dislodged from

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TABLE 1 *Histological Changes in Lymph Node Fragments of 300L and Underlying Gelatine Matrix in Long Term Culture*

Weeks in culture	Observations: Lymph node explants	Observations: Spongostan
1	Slight central necrosis with pycnotic lymphoid cells General structure preserved	No outgrowth
2	Moderate central necrosis Viable lymphoid and reticulum cells in periphery	Lymphoid cells and fibroblastlike cells begin to invade the sponge matrix The lymphoid cells mainly blastoid, are loosely attached to the fibroblasts
3	Central proliferation of fibroblastoid cells Well preserved lymphoid and reticulum cells in the periphery	The mixture of fibroblasts, lymphoid and reticulum-like cells begins to organize as a tissue
4	Only few lymphoid cells left in the partially necrotic centre Blastoid as well as mature lymphoid cells together with some reticulum like cells can be seen as small islands in the periphery	Cells continue to invade the sponge matrix
5	As in week 4	Development into an organotypic structure completed
6 7	As in week 4	As in week 5
8 9	Increased numbers of blastoid lymphoid cells in the periphery	
9½	Peripheral islands of lymphoid reticulum like cells larger Centre still fibroid	As in week 5 plus intense lymphoblastoid peripheral proliferation

ment was nondescript but by week 5 fibroblasts reticulum cells and lymphoid cells had organized themselves to a structure resembling a lymph node *in vivo* Fibroblasts covered the gelatin meshwork Closely apposed to these, reticulum cells were observed to be intermingled with lymphoid cells which histologically mainly conformed with lymphoblasts

After week 8 coinciding with the release of large numbers of lymphoblasts into the medium (*i e* the lymphoblastoid *transformation*) the number of the now mainly immature lymphoid cells increased considerably in the periphery of the explant and the piece of Spongostan (Fig 5–6)

DISCUSSION

The Spongostan (gelatin foam) grid method described here seems to be better than previous techniques for cultivation of lymphoid tissue as judged by a success rate of virtually 100 *per cent* (*Nilsson* 1971) The present histological study has shown only insignificant necrosis even after almost 3 months in culture and a preservation of the basic lymph node architecture In earlier systems where Spongostan was omitted a large proportion of lymphoid cells were lost either because of overt necrosis or failure of maintenance of lymph node structure with consequent loss of cells to the medium (*Nilsson et al* 1968) An additional improvement has been the exchange of Eagle's MEM for *Ham's F-10* medium

With the old method three fairly distinct phases of growth could be distinguished With the Spongostan technique, the borders between these became obliterated This was

the original explant were followed and alterations among these formed the morphological basis of the lymphoblastoid transformation (*Pontén* 1967, *Nilsson et al* 1968) A rather complex series of events was described, involving a primary lymphoid cell production phase, a secondary phase with predominance of fibroblast-like cells and a tertiary stage (lymphoblastoid transformation) with recurrent, and this time permanent growth of immature lymphoid cells This communication examines the histology of the explant itself to see how the stages outlined above correlate with alterations within the cultivated biopsy Is the hiatus between the initial and permanent production of lymphoid cells due to complete destruction of the originally present lymphoid elements or can the delay be explained by adaptation to conditions *in vitro*?

MATERIAL AND METHODS

A lymph node, 300L, was removed from the groin of a 37-year old female operated on for a varicose leg It was immediately placed in tissue culture medium and brought to the laboratory where it was cut up into small (1–2 mm³) fragments Five to six fragments were placed on the surface of a piece of gelatin foam (Spongostan, AB Ferrosan, Malmo Sweden) measuring 15 × 15 × 3 mm lying on top of a stainless steel platform covered by a lens paper Each grid was placed in a 50 mm Falcon plastic Petri dish The level of medium was adjusted to place the piece of gelatin foam at the gas liquid interphase

Culture medium Nutrient medium F 10 (Grand Island Biol Comp New York USA) supplemented with post natal calf serum (10 per cent) penicillin (100 IE/ml), streptomycin (50 µg/ml) and amphotericin B (1 25 µg/ml) was used

Cultivation and morphological methods Cultures were incubated at 37° C in a humidified 5 per cent CO_2 *in air atmosphere* At intervals, part of the Spongostan matrix, together with a lymph node fragment, was carefully excised from the grid culture with a scalpel The material removed was fixed in 10 per cent formaldehyde embedded in paraffin, sectioned and stained according to Van Gieson, with Haematoxylin Eosin and Methyl Green Pyronine

The cultures were observed twice a week through a Leitz inverted microscope Stained sections were examined in Leitz Ortholux microscope and photographed with a Leitz Orthomat camera

RESULTS

Observations of Cells Released from the Explants

During the first week many small lymphocytes and a few larger macrophagelike cells dropped down from the grid The latter attached to the bottom of the Petri dish but did not proliferate and eventually disappeared in 1–2 weeks The production of lymphoid cells, in small degenerating clumps or as separate cells, gradually diminished during the first month in culture These cells only rarely attached to the bottom of the dish and very few mitoses were observed

Increasing amounts of fibroblast like cells attached to the bottom of the plastic dishes 3–4 weeks after explantation

After two months the number of released lymphoid cells began to increase rapidly The cells were larger than those originally discharged and occurred in clumps estimated to contain up to several scores of cells These lymphoblastoid cells had the morphological characteristics reported before (*Ponten* 1967, *Moore et al* 1968) and attached to the surface of fibroblasts (peripolesis) The three stages described were typical of the lymphoblastoid transformation process

Histology of Explants and Spongostan Matrix

The histological changes have been divided into those occurring within the explant itself and those caused by penetration of cells into the surrounding Spongostan matrix (Table I and Fig 1–8)

Within the interior of the explant moderate necrobiotic alterations were seen after one week Necrosis progressed and the centre was slowly replaced by fibrous tissue The periphery contained viable reticulum and lymphoid cells at all times (Fig 2 3, 4, 5, 7 and 8) The lymphoid cell population consisted at the start mainly of mature cells These were gradually replaced by blastoid cells which from week 6 were the predominant cell type

Two weeks after explantation lymphoid blast cells and fibroblasts began to invade the sponge matrix (Fig 1) At first the arrange-

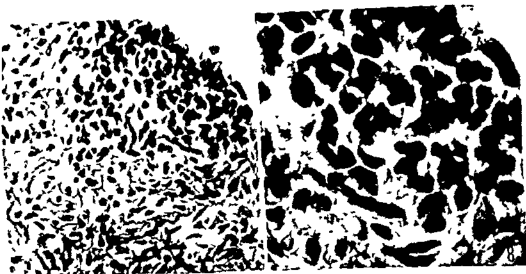


Fig 7 Lymph node section at 9½ weeks Note the increased number of blastoid cells in the periphery compared to Fig 3 and 4 × 100 Van Gieson.

Fig 8 Peripheral part of the lymph node explant at 9½ weeks Large numbers of lymphoblasts in close relationship with fibroblasts located deeper in the lymph node Note the morphological similarity with cells found in Fig 2 × 350 Van Gieson

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Fig 1 Lymph node (left) at 2 weeks with viable *and pycnotic lymphoid cells Increased* number of fibroblasts in the stroma Invasion of lymph node cells into the interstices of Spongostan (right) × 90 Van Gieson

Fig 2 Periphery of a 3 week-old lymph node fragment Mature and blastoid lymphoid cells and reticulum cells intermingled with fibroblasts × 350 Van Gieson

Fig 3 Peripheral part of a 6-week-old explant. Predominance of fibroblasts and blastoid lymphoid cells Note resemblance to Fig 2 × 350 Van Gieson

Fig 4 Lymph node section at 7 weeks The centre of the explant is necrotic (extreme upper left) Note the peripheral location of viable cells × 90 Van Gieson

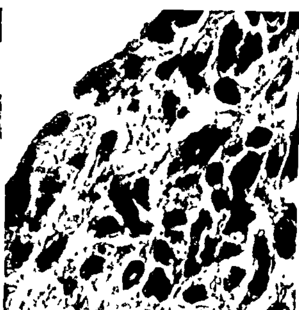
Fig 5 8-week-old lymph node explant (peripheral part) with an increased number of lymphoblasts in the periphery and on the surface Compare with Fig 3 Rapidly proliferating lymphoblasts were observed in the culture at this time (lymphoblastoid transformation) × 90 Van Gieson

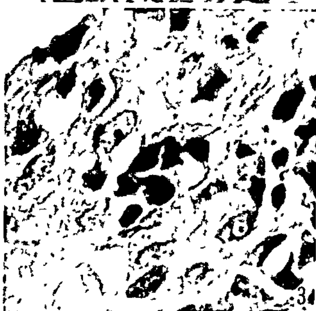
Fig 6 Section of 9½ week-old Spongostan The cells proliferate particularly in the periphery × 30 Van Gieson

most clear in the regularly examined sections of the explants Throughout the observation time, viable lymphoid cells of varying maturity were found The general picture changed little Even at the time when rapidly proliferating lymphoblasts appeared in the medium, the histological findings were almost the same as in sections obtained 4 weeks earlier The only recognizable change was an enlargement of lymphoid cell 'islands" in the periphery of the explants and the gradual change of the l

such blastoid cells morphologically similar to permanent lymphoblasts, were present in the histological sections at all stages of the establishment it is suggested that lymphoblastoid transformation is not a transformation of fibroblasts *in vitro* into infinitely growing lymphoblasts as suggested by *Benyesh Melnick et al* (1963), but a selection and/or adaptation *in vitro* of lymphoid cells *present in the biopsy* at the time of explantation In the studies of lymph node, bone

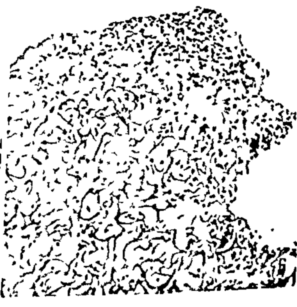

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TESTICULAR CHANGES IN ASSOCIATION WITH MALFORMATION OF THE CENTRAL NERVOUS SYSTEM AND MENTAL RETARDATION

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The testes were studied microscopically in 15 severely mentally retarded men with cerebral malformations They ranged from 19 to 71 years in age The testicular picture was normal in 8 In 3 there were moderate or severe testicular changes of the type usually classified as being of extrahypophyseal origin In 1 there were hardly any demonstrable Leydig cells in an adult testis and in 1 the testes were of infantil type In both these cases there was wide spread injury to the hypothalamus or structures associated with the hypothalamus In 2 men the picture of the testes was partly of infantile type and partly of adult type with hyper plasia of interstitial cells In both cases there were malformations of different organs and signs of endocrine disorders These two men showed extensive cortical cerebral malformations It is possible that the picture of the testes may be explained by injuries of the cerebral cortex with consequent disturbed stimulation of the testes

Degenerative processes in the central nervous system are often seen in association with testicular changes without coexisting demonstrable hypophyseal lesions (*Vague & al* 1963) A direct relationship is therefore assumed between lesions of the CNS and those of the testes and the condition is regarded as a combined neuro-germinal degeneration

It is well known that pathological processes of the hypothalamus can cause testicular changes in man (*Cecil & Loeb* 1961, *Morsier &Gauthier* 1963) but it is not known whether lesions in other parts of the brain can produce testicular changes In animals, it has been possible to produce changes in the testes by damaging the cortex (*Soulairac & Soulairac* 1963, *Marescotti & al* 1961)

It was therefore thought worth while to search for testicular abnormalities in men with serious mental retardation and demonstrable changes in the brain and try to find out whether a correlation exists between the lesions of the CNS and the picture of the testes

MATERIALS AND METHODS

The material consisted of 15 severely mentally retarded men, aged 19 to 71 (Table 1) Cases with malformations and anoxic injuries of the central nervous system were studied Those with metabolic, degenerative or generally debilitating diseases such

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marrow- and spleen explants in this laboratory employing the grid organ culture (*Nilsson et al* 1968 and *Nilsson* 1971) and by *Sinkovics et al* (1967) using monolayer cultures, fibroblasts were never observed to transform into lymphoblasts

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TABLE 1 *(Cont)*

Case no	Age	Ment	CNS diagnosis	Testicular picture	Remarks
14	18	I	Widespread cortical dysplasia and micropolygyria	Partial loss of germinal epithelium, immature tubules hyperplasia of interstitial cells	Twin brother retarded Small malformed penis Gynecomastia
15	25	0	Severe perinatal anoxic encephalopathy with brain atrophy including hypothalamus Epileptic encephalopathy	Immature testes	

as cancer or chronic infectious processes were excluded Except for few cases of the latter type these cases represent a consequtive autopy series of men dying during a 2 year period in a hospital for severely mentally retarded

Gross examination at autopsy included *inter alia* the central nervous system testes thyroid adre

bedded in paraffin sectioned and stained with haematoxylin-eosin van Gieson Mahon Nissl sil

were judged as being of adult type and in one case of infantile type The testes of adult type were classified according to the number and appearance of Leydig cells into 2 groups namely 1/ testes with normal Leydig cells and 2/ testes with abnormal Leydig cells This classification was based particularly on the number of Leydig cells A case was regarded as normal if the number of Leydig cells was normal and most of the cells were of normal appearance Only cases with an *absolute* increase or decrease in number of Leydig cells were regarded as abnormal This group did not include cases with a relative increase of the number of Leydig cells due to pronounced tubular fibrosis

The group with normal Leydig cells was subdivided according to the appearance of the tubules into 3 groups mild tubular degeneration *i e* at least two thirds of the tubules showed what may be regarded as normal spermiogenesis moderate tubular degeneration *i e* one third to two thirds of the tubules showed what may be regarded as normal spermogenesis, severe tubular degeneration *i e* less than one third of the tubules showed what may be regarded as normal spermiogenesis

17*

RESULTS

Testes with Normal Leydig Cells

Mild tubular testicular degeneration was found in 8 men aged 19, 24, 28, 33, 49, 57, 58 and 71 (Nos 1–8) There was hypospadia in 1 case (3) and hyperplasia of the prostate in one (4) The secondary sexcharacteristics were otherwise normal Urethral atresia was found in one case (3) Hypoplasia of the thyroid was demonstrated in two cases (3, 7), while the endocrine organs were otherwise normal In all cases the testes lay in the scrotum, they were of normal size or somewhat small

The CNS changes in this group consisted of scarring due to old infarction, anoxia and healed encephalomyelitis in restricted areas in 3 cases (6–8) In 4 cases there were cortical malformations (mild cortical dysplasia or micropolygyria in small areas but no heterotopias (1, 3–5) and unilateral multifocal micropolygyria with heterotopias and malformation of the callosal body in one case (2) The mild dysplasia was characterized in these cases, as in the other cases, by an increase in the width or irregular outline of the cortex, indistinct or undemonstrable lamination, malplaced or maloriented and often immature nerve cells Heterotopias in this and other groups of cases consisted of numerous scattered nerve cells in the white substance or islands of grey substance

Moderate tubular testicular degeneration

TABLE 1 *Material*

Case no	Age	Ment age	CNS diagnosis	Testicular picture	Remarks
				Normal Leydig Cells	
1	19	I	Focal mild cortical dysplasia. Subcortical gliosis	Slight tubular degeneration	
2	24	II	Unilateral multifocal micro polygyria, heterotopias and malformation of callosal body	Slight tubular degeneration	
3	28	IV	Focal mild cortical dysplasia Signs of healed encephalo myelitis	Slight tubular degeneration	Thyroid gland small Atretic, hypospadic uretra.
4	33	I	Focal micropolygyria and mild cortical dysplasia	Slight tubular degeneration	Prostatic gland small
5	49	II	Slight cortical dysplasia	Slight tubular degeneration	
6	57	V	Signs of healed encephalitis and epileptic encephalopathy	Slight tubular degeneration	
7	58	IV	Central atrophy Signs of healed encephalomyelitis (poliomyelitis and/or pertussis) Anoxic encepha lopathy	Slight tubular degeneration	Thyroid gland small
8	71	VI	Old infarction Senile ence-phalopathy No malformations	Slight tubular degeneration	Mother and 3 siblings retarded
9	20	0	Focal micropolygyria, mild cortical dysplasia, heterotopias Anoxic encephalopathy	Moderate tubular degeneration	Thyroid gland small
10	60	II–III	Widespread mild cortical dysplasia, heterotopias Benign ependymoma of amygdala Brain atrophy incl fornix and mammillary bodies	Moderate tubular degeneration	Thyroid gland small
11	64	I	Perinatal anoxic encephalo pathy with brain atrophy	Severe tubular degeneration	Skeletal malfor mation
				Abnormal Leyding cells	
12	48	III	Widespread cortical dysplasia, heterotopias Sclerosis of pal lidum and amygdala Anoxic encephalopathy	Almost total loss of germinal epithelium, thickening of tubular wall interstitial fibrosis a few Leydig cells	4 siblings retarded
13	44	II	Widespread cortical dysplasia most pronounced in the frontal lobes which were hypoplastic, heterotopias Mal formation of base of skull and of falx cerebri	Reduced spermio genesis hyalin thicken ing of tubular wall, tubular fibrosis, im mature tubules, hyper-plasia of interstitial cells	Skeletal malf, hypogonadism dwarfism malf of penis and gyne comastia (X lin ked recessive syndrome)

TABLE 1 *(Cont.)*

Case no	Age	Ment	CNS diagnosis	Testicular picture	Remarks
14	18	I	Widespread cortical dysplasia and micropolygyria	Partial loss of germinal epithelium, immature tubules, hyperplasia of interstitial cells	Twin brother retarded Small malformed penis Gynecomastia
15	25	0	Severe perinatal anoxic encephalopathy with brain atrophy including hypothalamus Epileptic encephalopathy	Immature testes	

as cancer or chronic infectious processes were excluded Except for few cases of the latter type these cases represent a consequtive autopy series of men dying during a 2 year period in a hospital for severely mentally retarded

Gross examination at autopsy included *inter alia* the central nervous system, testes, thyroid, adrenals [illegible] material was embedded in paraffin, sectioned and stained with haematoxylin-eosin, van Gieson, Mahon, Nissl, silver impregnations and the hypophyses with a modified Mallory's stain (*Kernohan* 1931) The material was classified according to the histological picture of the testes In 14 of the cases the testes were judged as being of adult type, and in one case of infantile type The testes of adult type were classified according to the number and appearance of Leydig cells into 2 groups namely 1/ testes with normal Leydig cells and 2/ testes with abnormal Leydig cells This classification was based particularly on the number of Leydig cells A case was regarded as normal if the number of Leydig cells was normal and most of the cells were of normal appearance Only cases with an *absolute* increase or decrease in number of Leydig cells were regarded as abnormal This group did not include cases with a relative increase of the number of Leydig cells due to pronounced tubular fibrosis

The group with normal Leydig cells was subdivided according to the appearance of the tubules into 3 groups mild tubular degeneration, i e at least two thirds of the tubules showed what may be regarded as normal spermiogenesis moderate tubular degeneration i e one third to two thirds of the tubules showed what may be regarded as normal spermiogenesis, severe tubular degeneration, i e less than one third of the tubules showed what may be regarded as normal spermiogenesis

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8	71	VI	Old infarction Senile encephalopathy No malformations	Slight tubular degeneration	Mother and 3 siblings retarded
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11	64	I	Perinatal anoxic encephalopathy with brain atrophy	Severe tubular degeneration	Skeletal malformation
				Abnormal Leyding cells	
12	48	III	Widespread cortical dysplasia, heterotopias Sclerosis of pallidum and amygdala Anoxic encephalopathy	Almost total loss of germinal epithelium thickening of tubular wall interstitial fibrosis, a few Leydig cells	4 siblings retarded
13	44	II	Widespread cortical dysplasia most pronounced in the frontal lobes which were hypoplastic, heterotopias Malformation of base of skull and of falx cerebri	Reduced spermiogenesis hyalin thickening of tubular wall tubular fibrosis, immature tubules hyperplasia of interstitial cells	Skeletal malf, hypogonadism dwarfism malf of penis and gynecomastia (X linked recessive syndrome)


1
2
3
4

was seen in 2 men, aged 20 and 60 The chromosome pattern was examined in one (9) and showed nothing remarkable The secondary sex characteristics were normal In both cases there was hypoplasia of the thyroid, while other endocrine organs were normal In both cases the testes were situated in the scrotum and were somewhat small

The brains showed focal micropolygyria, mild cortical dysplasia with heterotopias and anoxic encephalopathy in one case (9) and widespread mild cortical dysplasia with heterotopias in one case (10) together with a benign ependymoma of amygdala and brain atrophy involving also the fornices and mamillary bodies

Severe tubular testicular degeneration was found in one man, aged 54 The testes were small and situated in the scrotum He had skeletal atrophy, a flattened thorax with pronounced scoliosis and club foot The secondary sex characteristics were normal, as were the endocrine organs

The picture of the CNS in this case was that of a perinatal anoxic encephalopathy with cerebral atrophy, without hypothalamic involvement

Testes with Abnormal Leydig Cells

This group was subdivided into cases with reduction of the number of Leydig cells and cases with hyperplasia of Leydig cells

Reduction of the number of Leydig cells was seen in 1 case (12), a man aged 48 who had four mentally retarded siblings Secondary sex characteristics normal Hypophysis, thyroid and adrenals normal The brain showed widespread cortical dysplasia, heterotopias sclerosis of the globus pallidus and nucleus amygdale as well as diffuse anoxic encephalopathy The testes were of normal size and were situated in the scrotum Microscopically most tubules showed a slightly reduced diameter They were lined by Sertoli cells and most tubules contained spermiogons though only a few A few tubules had single spermiocytes but no spermids Many cells were degenerated The tubular wall was moderately sclerosed The interstitial tissue showed fibrosis and contained a small number of Leydig cells Most Leydig cells were small and degenerated (Fig 1)

Hyperplasia of Leydig cells was seen in two cases

Case 13 A man, aged 44, of stunted growth, obese, with gynecomastia, scanty axillary and normal pubic hair growth Fingers and toes were short The 2nd and 3rd toes had grown together the base of the skull was deformed The chromosomal pattern was normal The penis was short There was thyroid hypoplasia and small parathyroid adenoma (Borjeson Forsman Lehmansyndrome)

The picture of the brain was characterized by heterotopias and diffuse cortical dysplasia involving all lobes but most pronounced in the frontal lobes which were hypoplastic The testes were small, soft and situated in the scrotum Microscopically many tubules were totally sclerosed Patent tubules varied in size and were slightly to strongly sclerosed They were lined by Sertoli cells and a varying number of spermiogons and spermiocytes Many cells were degenerated Some tubules were immature There was hyperplasia of Leydig cells, varying in size from small to large Some Leydig cells were fusiform, other polygonal Many of the largest cells had a pale vesicular cytoplasm (Fig 2)

Case 14 A man, aged 18, with adiposity and gynecomastia A twinbrother was men

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Fig 1 Case 12 Man aged 48 Tubular diameter slightly reduced Tubules lined by *Sertoli cells* spermiogons and a few spermiocytes Many degenerating cells Fibrous thickening of the tubular wall Slight interstitial fibrosis A few small, degenerating Leydig cells and round cells in the interstitial tissue *Haematoxylin eosin* × 130

Fig 2 Case 13 Man aged 44 Totally sclerosed tubules and some patent tubules showing incomplete spermiogenesis Hyperplasia *of Leydig cells* Haematoxylin eosin × 130

Fig 3 Case 14 *Man aged 18* Most tubules lined only by Sertoli cells Hyperplasia of Leydig cells Haematoxylin eosin × 130

Fig 4 Case 15 Man aged 25 Small immature tubules lined by cells with either dark or light *nuclei Interstitial* tissue immaturely cellular Leydig cells present Haematoxylin eosin × 210

Microscopic examination, however, revealed the hypophysis to be normal in both cases A normal microscopic picture does not of course exclude a functional disorder

It is known that stimulation of the gonads by gonadotrope hormones can fail in persons with morphologically preserved hypophysis (*Lloyd* 1963) In such cases it has been possible to demonstrate injury to the hypothalamus Experimental investigations have however not been able to define a distinct centre in the hypothalamus for the gonadotropic regulation of the gonads but rather an extensive hypothalamic system (*Flerko* 1963 *Ahren* 1963) Clinical and experimental observations also suggest that injury to both the anterior and posterior hypothalamus and tuber cinereum can influence the gonads

In the present material is was possible to demonstrate substantial lesions of the hypothalamus in only 1 case (15) in which sclerosis and atrophy of mainly one half of the hypothalamus was demonstrated This sclerosis and atrophy was part of a predominantly unilateral anoxic cerebral atrophy Milder anoxic injury was also demonstrated in the opposite hemisphere It was in this case that the testes were of infantile type Injury to the hypothalamus may be the cause of the deficient gonadotropic stimulation of the testes in this case Changes in structures associated with the hypothalamus were seen in 1 case (12) Here the pallidum and amygdala were the sites of gliosis with atrophy Also in this case there were signs of deficient gonadotropic stimulation of the testes The number of Leydig cells was markedly reduced

In a further case (10) there were milder changes in structures associated with hypothalamus Here sclerosis and atrophy of fornices were demonstrated as well as an ependymoma of one amygdaloid nucleus In this case there was moderate tubular degeneration while Leydig cells were normal Testicular changes in this case were thus classified as not being due to deficient gonadotropic stimulation

The picture of the testes which is characterized by tubular fibrosis presence of immature tubules and hyperplasia of the interstitial cells is encountered in Klinefelter's syndrome (*Klinefelter* & *al* 1962) The testicular lesion in these cases is belived to be of genetic origin This picture of the testes can, however be produced by non genetic factors (*Raboch* 1963) Testicular changes of this appearance have been seen following experimental injuries to the cerebral cortex in the rat (*Marescotti* & *al* 1961, *Soulairac* & *Soulairac* 1963)

In the present material the testes in 1 case (13) showed a microscopic picture characterized by hyperplasia of interstitial cells tubular fibrosis and immature tubules Thus in several respects the picture in this case resembled that seen in Klinefelter s syndrome The chromosomal pattern was however, normal In this case there were, however also skeletal anomalies stunted growth hypogonadism malformed penis and gynecomastia The symtom complex in this case is known to be sexlinked and recessive (*Borjesson* & *al*, 1961, *Baar* & *Galindo* 1965) In a further case (14) microscopic examination showed the testes to be partly of infantile and partly of adult type The interstitial cells showed hyperplasia Also in this case there were anomalies and other signs of disturbed endocrine function in the form of a malformed penis and gynecomastia

The cause of the testicular picture in these two cases might be sought primarily in the testis possibly of the nature of a genetic injury It is not less likely however that the testicular changes were due to deficient stimulation by the gonadotrope hormones These two cases also displayed other anomalies of endocrine and sex organs The cause of this defective stimulation might perhaps be sought in injuries to the cerebral cortex which were found in these cases According to *Nauta* (1963) much suggests that the cortical structures especially in the temporal and frontal lobes exert a control of the hypothalamus in all primates

Cerebral cortical changes of principally the same type as those seen in these 2 cases were demonstrated also in cases 1–8 where the tes

tally retarded The penis was hypoplastic with a tendency to hypospadia The pubic hair was of somewhat feminine type Hypophysis adrenals and thyroid normal The brain showed *widespread cortical* dysplasia and micro polygyria involving all lobes Both testes were *small the right one located* in the scrotum and the left in the groin Microscopically the right testis showed mostly *adult tubules but* reduced in size Most tubules contained no germinal cells and were lined by Sertoli cells only A few showed spermiogenesis The tubular wall was normal or slightly sclerosed Interspersed among adult tubules were groups of immature tubules The Leydig cells showed hyperplasia (Fig 3) They varied in size Some were fusiform though most were polygonal The left testis *contained mostly immature* tubules A few tubules were adult but reduced in size and lined by Sertoli cells only There was hyperplasia of Leydig cells

Infantile Testes

Infantile testes were seen in one case (15) a man aged 25 with normally developed secondary sex characteristics Chromosome pattern was normal Hypophysis thyroid and adrenal normal The brain showed mainly right sided severe *atrophy* involving also the hypothalamus and anoxic perinatal and epileptic encephalopathy The testes were small and situated in the scrotum Microscopically the testes contained immature tubules lined by cells with elongated dark nuclei and round cells with larger nuclei The interstitial tissue *was rich in cells many were fibrocyte* like some resembled Leydig cells (Fig 4)

In no case were there any hypophyseal alterations

DISCUSSION

This material had been obtained at autopsy from 15 adult males with serious mental retardation since birth or the first year of life Changes were *demonstrated in the CNS* and consisted of malformations and anoxic cerebral lesions *The picture of the testes* was judged as normal in 8 of these 15 cases In these 8 cases mild degenerative changes confined to the tubules were demonstrated In the remaining 7 the picture of the testes was pathological In 3 of these the changes were confined to the tubules while in 3 changes were demonstrated both in the tubules and in the interstitial tissue In one the testes were of infantile type

Degenerative changes in the testes are usually classified as hypophyseal or extrahypophyseal in origin (*Nelson* 1953) In testicular degeneration of extrahypophyseal type the injuries are primary in the testes Testicular degeneration of hypophyseal type is due to insufficient stimulation by gonadotrope hormones Characteristically these cases show a reduction in the number of Leydig cells

Testicular degeneration of extrahypophyseal origin is believed to be due to a variety of factors partly of local nature such as trauma circulatory disorders heat inflammation and partly general diseases such as chronic infections nutritional disorders vitamin deficiency toxic effect (*Oberndorfer* 1931)

Mild degenerative changes in some tubules are seen in all testes and may therefore be regarded as normal Moderate or severe degenerative testicular changes of extrahypophyseal origin were seen in 3 cases (9 10 11) In these cases the Leydig cells were normal while the tubules showed degenerative changes of *pathological extent Local factors* or general disorders as an explanation of this picture of the *testes* could however not be shown in these cases

Testicular degeneration due to insufficient stimulation by gonadotrope hormones may be caused by pathological processes in the hypophysis or its surroundings *it may be of* functional origin or idiopathic (*Nelson* 1953) *If the gonadotrope* stimulation becomes deficient before puberty maturation of the testes is inhibited

The present material included one case (1?) with hardly any Leydig cells in an adult testis *In a 25 year old man (15)* the testes were of infantile type In these 2 cases the testicular changes as judged from the morphological picture were of hypophyseal type

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MUCOPOLYSACCHARIDOSIS TYPE III (SANFILIPPO'S SYNDROME)

Histochemical Examination of the Eyes and Brain with a Survey of the Literature

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In a clinically diagnosed case of Sanfilippo's syndrome (Mucopolysaccharidosis type III) histopathological examination of the eyes revealed accumulation of a *granular material* mainly in the sclera and to a lesser degree in the cornea and ciliary body Similar scleral changes have previously been reported in various types of mucopolysaccharidosis in six cases only, and ciliary body changes in two cases *only* *It is emphasized that* ocular involvement including corneal changes may be found histopathologically even if not clinically detected The main histopathological findings in the cerebrum and cerebellum were ballooning of ganglion cells similar to the findings in other types of storage disease By applying a battery of histochemical methods to the ocular and cerebral material the findings indicated 1) a difference between the stored material in the brain and in the eyes 2) that the stored material in the ganglion cells of the brain was probably of a ganglioside nature and 3) that the material in the eyes was consistent with an acid mucopolysaccharide, undoubtedly sulphated and probably rich in heparitin sulphate These findings are compared with the suggested biochemical changes and the correlation between these and the morphology is discussed The writer is in favour of the theory of a basic enzyme defect leading to accumulation of the granular material in the cells Addition of normal and pathological cytoplasmic constituents are probably responsible for the difference in histochemical findings, especially in fibrocytes and ganglion cells as revealed by the present study The eyes have not previously been histopathologically or histochemically examined in Sanfilippo's syndrome

Histopathological examination of eyes, brain and other organs has been carried out in several cases of Hurler's syndrome, often under the heading of gargoylism Histochemical examination in these cases has often led to varying results and stainings for acid mucopolysaccharides (AMP) often to negative results (See under discussion)

The aim of the present work was to study histopathologically and histochemically the eyes and brain in a case of Sanfilippo's syndrome a rather infrequent type of mucopolysaccharidosis, and to compare the findings with previously published material and with the biochemical defect Special interest has been given to study of the ocular changes,

Received 28 x 70

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ticular picture was judged as normal Among these cases, however, the cerebral damage was less extensive in cases 1–5 and only slight or minimal in cases 6–8 This group also had the highest mental age rating The group showing moderate to severe tubular degeneration (9–11) had more pronounced and disseminated changes and also a lower mental age rating Those with abnormal Leydig cells (12–14) and immature testes (15) displayed the gravest cerebral changes and profound mental incapacity

In no case could the hypophysis on morphological grounds be held responsible for a disturbed hormonal stimulation of the testes

The number of testicular abnormalities found in this small but practically consecutive series of autopsies by far exceeds the expected frequency judging from a large consecutive autopsy series without selection regarding CNS diseases or mental retardation, counting also degenerative and debilitating diseases which might more directly influence the testes

The results of the study of this small series did not permit conclusions regarding a cerebral suprahypophyseal and in particular cortical control of the testes An investigation of a larger series might perhaps yield information on this point

The results of the investigation, however, on the basis of the disproportionately high frequency of testicular abnormalities together with lack of hypophyseal changes and the correlation between severity of CNS affliction and testicular abnormality, appears to support the assumption of such a cerebral control over testicular function and morphology

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Fig 3 The face of the patient at the age of 4 Note the mild gargoyle features and the clear corneas

Ophthalmological examination revealed no abnormalities and especially no corneal opacity (Fig 3) at several examinations but according to the record slit lamp examination had not been carried out Ophthalmoscopy on the other hand had always revealed normal transparency of the refracting med a The hearing was found to be normal The EEG showed low frequency activity of 1–2 hz and sharp waves bifronto-temporally Psychological examination showed a decreasing I Q from nearly normal at 3 years to about 30 at 5 years of age At the age of five X rays showed a development of the bone nuclei corresponding to 3 years of age scaphocephalia and premature synostosis of the sagittal suture as well as deformation of the 2nd 4th and 5th lumbar vertebrae with moderate lumbar kyphosis (Fig 2) The sagittal diameter of the affected vertebrae was smaller than that of the normal vertebrae and in the anterior surface a deep impression was observed The phalangeal and metacarpal bones were broad and short resembling the changes seen in Hurler's syndrome

The heart examination and routine laboratory tests were normal Paper electrophoresis of urine showed an increased fraction of acid mucopolysaccharides (AMP) but differentiation between chondroitin sulphate and heparitin sulphate could not be established by the method applied

The patient was at last transferred to an institution for mental deficiency where he suddenly died at the age of 8 years after several attacks of bronchitis (Ebberodgaard Rec no B 1885) *The autopsy* (*J Voigt*, J nr 1 16/68) showed incomplete pulmonary atelectasis perivascular fibrosis of the myocardium, fibrosis of the bicuspid valves, light interstitial myositis and hepato- and splenomegalia Frozen sections of liver tissue showed a few fine lipid droplets in the cells and sporadic PAS positive material interstitially, not in the cells It was concluded that the findings were consistent with a slight degree of Hurler's syndrome

Examination of the brain (Psyk lab no 5826 signed *J Bestle*) showed perivascular spaces filled with a reticulum of collagen fibrils, and PAS-positive and slightly sudanophilic material in the ganglion cells of the cerebrum and cerebellum The diagnosis was [illegible] (gargo[illegible] analysi[illegible] Sept 1[illegible] a significant reduction of myelin lipid was found (cerebrosides sulphatides cholesterol phosphatidyl serine, and phosphatidyl inositol) characteristic of a slightly demyelinating process Thin layer chromatography showed slight relative increases in di and trisialogangliosides No ganglioside of Tay Sachs type was found

A revision by *Melchior* (42) of the clinical and laboratory data led to the conclusion that San filippo s syndrome was the most probable diagnosis (see discussion)

MATERIAL AND METHODS

The eyes (Ophth Path Lab nos 343/68, 344/68) and brain (Ophth Path Lab no 44/70) were obtained post-mortem and fixed in 4 per cent buffered neutral formaldehyde for 24 hours at 4° C The brain however, had been kept in formalin for several months before histochemical examination was performed Usable tissue from other organs was unfortunately not obtainable at this time Routine paraffin technique had been employed in processing the eyeballs as well as frozen sections of the formalin fixed tissue

Pieces of tissue of the motor cortex and cerebellar cortex were embedded in gelatin (5) before frozen sections were made Paraffin sections stained with Haematoxylin-eosin PAS, PAPS and AB, as well as paraffin sections of cerebellar and motor cortex tissue from an anoxic brain, were likewise examined for the sake of comparison

The staining reactions including their abbreviations with a reference to methods as well as to their significance are to be seen in Tables I and 2 When no special reference is made the staining is according to methods employed by *Pearse* (49)

Enzyme digestion was performed with Diastase[1],

[1] Diastase 2800 E/g Merck, Darmstadt no 3013

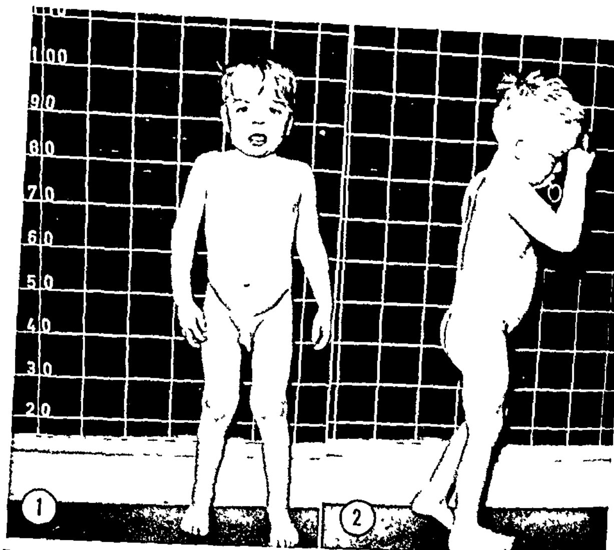


Fig 1 and 2 Front- and side view of the patient at the age of 4 Note the mild gargoyle features and the moderate lumbar kyphosis and no gibbus

as histopathological or histochemical examinations of these have not previously been carried out in this type of mucopolysaccharidosis

CASE REPORT

(Dronning Louises Bornehospital Rec no 877/65) A 3 year old boy was first seen because of arrested mental development His father and uncle both of American origin, had a facial resemblance to the patient, but were healthy The mother was healthy No consanguinity was present He was the second of three children His two brothers were healthy The pregnancy was normal He was asphyctic for 5 or 10 minutes after delivery, which was otherwise normal He was fully developed At the ages of 3 and 5 weeks he had a right and a left inguinal herniotomia performed Both hernia recurred at the age of five and a left sided reoperation together with an umbilical herniotomia were performed The mental development was normal until about 3 years of age

The diagnosis at the first admission to the hospital was asphyctic encephalopathy, but a mucopolysaccharidosis was suspected This latter diagnosis was supported at several admissions during the following 5 years by the following evidence The appearance of the boy (Fig 1, 2 and 3) was that of a strong short necked boy with a scaphocephalic skull (Fig 2) and slight ocular hypertelorism The nose was short with a tendency towards saddle formation, the mouth large and the lips prominent (Fig 3) The feet and hands were plump and the distal phalanges of the fingers had a slight lateral deviation The extension of the elbow , hip , and knee joints was slightly limited so that he walked with knees and hips slightly flexed (Fig 2)



Fig 3 The face of the patient at the age of 4 Note the mild gargoyle features and the clear corneas

Ophthalmological examination revealed no abnormalities and especially no corneal opacity (Fig 3), at several examinations, but according to the record slit lamp examination had not been carried out Ophthalmoscopy on the other hand had always revealed normal transparency of the refracting media The hearing was found to be normal The EEG showed low frequency activity of 1–2 hz and sharp waves bifronto-temporally Psychological examination showed a decreasing I Q, from nearly normal at 3 years to about 30 at 5 years of age At the age of five, X rays showed a development of the bone nuclei corresponding to 3 years of age scaphocephalia and premature synostosis of the sagittal suture as well as deformation of the 2nd 4th and 5th lumbar vertebrae with moderate lumbar kyphosis (Fig 2) The sagittal diameter of the affected vertebrae was smaller than that of the normal vertebrae and in the anterior surface deep impre [illegible]

[illegible] were normal [illegible] outine laboratory showed an increased fraction of acid mucopolysaccharides (AMP), but differentiation between chondroitin sulphate and heparitin sulphate could not be established by the method applied

The patient was at last transferred to an institution for mental deficiency, where he suddenly died at the age of 8 years after several attacks of bronchitis (Ebberodgaard Rec no B 1885) *The autopsy* (*J Voigt*, J nr I 16/68) showed incomplete pulmonary atelectasis, perivascular fibrosis of the myocardium, fibrosis of the bicuspid valves, light interstitial myositis and hepato- and splenomegalia Frozen sections of liver tissue showed a few fine lipid droplets in the cells and sporadic PAS positive material interstitially, not in the cells It was concluded that the findings were consistent with a slight degree of Hurler's syndrome

Examination of the brain (Psyk lab no 5826, signed *J Bestle*) showed perivascular spaces filled with a reticulum of collagen fibrils, and PAS-positive and slightly sudanophilic material in the ganglion cells of the cerebrum and cerebellum The diagnosis was lipo-mucopolysaccharidosis cerebri (gargoylism Hurler's syndrome) By microchemical analysis of formalin fixed brain tissue (*J Clausen* Sept 1970) a significant reduction of myelin lipid was found (cerebrosides, sulphatides cholesterol, phosphatidyl serine, and phosphatidyl inositol) characteristic of a slightly demyelinating process Thin layer chromatography showed slight relative increases in di and trisialogangliosides No ganglioside of Tay Sachs' type was found

A revision by *Melchior* (42) of the clinical and laboratory data led to the conclusion that San filippo's syndrome was the most probable diagnosis (see discussion)

MATERIAL AND METHODS

The eyes (Ophth Path Lab nos 343/68, 344/68) and brain (Ophth Path Lab no 44/70) were obtained post mortem and fixed in 4 per cent buffered neutral formaldehyde for 24 hours at 4° C The brain, however, had been kept in formalin for several months before histochemical examination was performed Usable tissue from other organs was unfortunately not obtainable at this time Routine paraffin technique had been employed in processing the eyeballs as well as frozen sections of the formalin fixed tissue

Pieces of tissue of the motor cortex and cerebellar cortex were embedded in gelatin (5) before frozen sections were made Paraffin sections stained with Haematoxylin eosin, PAS, PAPS and AB, as well as paraffin sections of cerebellar and motor cortex tissue from an anoxic brain, were likewise examined for the sake of comparison

The staining reactions including their abbreviations with a reference to methods as well as to their significance are to be seen in Tables 1 and 2 When no special reference is made, the staining is according to methods employed by *Pearse* (49)

Enzyme digestion was performed with Diastase[1],

[1] Diastase 2800 E/g Merck, Darmstadt no 3013

pepsin[2], testicular hyaluronidase[3], and protease free *Vibrio cholerae* neuraminidase[4]

The diastase was applied at 37° C for 60 min and followed by PAS The other enzymes were used in solutions and concentrations as well as at exposure times according to *Quintarelli* (52) Control sections were incubated in buffer solutions without enzymes for the same periods and at the *same temperatures* After exposure of these enzymes the PAS reaction the metachromatic methods and the AB at pH 1 and 2 5 as well as the combined AF AB and HID AB methods were applied

RESULTS

The significant finding in the eyes was numerous closely placed uniform vacuoles or spatia, some apparently empty, but several containing a fine granular material slightly basophilic as seen in the haematoxylin eosin staining (Fig 4) or yellow in van Gieson and Sirius staining These changes were most *pronounced in the sclera while the corneal* stroma showed fewer, somewhat larger but more widespread and emptier spatia placed evenly in the stroma (Fig 5) and not especially beneath the epithelium in which no changes were found No large light cells or clusters of such cells were found beneath or in contact with the epithelium Similar spatia were observed in the ciliary body especially the ciliary processes (Fig 6) Their presence in the retina was doubtful Nearly all vacuoles had an eccentrically placed nucleus No doubt most of these vacuoles represented vacuolated fibrocytes In the cortex of cerebrum and cerebellum the significant finding was ballooning of ganglion cells, the Purkinje cells were especially distended (Fig 7) The cytoplasm of these cells was likewise finely granular

To investigate the nature of this granular material in fibrocytes and ganglion cells the staining and enzyme procedures referred to above were performed The results may be seen in Tables 1 2 and 4 The staining reactions were graded arbitrarily, and an unsuccessful or not performed reaction designated by

Regarding the results of the stainings after enzyme treatment, no significant difference was observed in exposed *sections and control* sections

It can be seen from Table 1 that no lipid material at all was found in the eyes while the material in the ganglion cells of the motor cortex and cerebellar cortex showed faint *reactions with the red Sudan dye Oil red O* Sudan black B, phosphomolybdic acid and a blue colour with Nile blue sulphate using *Cain's* technique while the copper phthalocyanin dye Luxol fast blue gave a significant blue staining of the granular material (Fig 8)

In Table 2 it may be seen that upon applying *pyridine extraction the* subsequent PAS reaction was greatly reduced often so much so that a moth eaten appearance of the cytoplasm was observed (Fig 9) The AB stainings were negative after this extraction Extraction with ether at room temperature did not change the PAS reaction or the AB stainings Dimedone blockade followed by PAS reduced the PAS reaction only slightly In several attempts, the modified PAS method of *Adams and Bayliss* gave so heavy a destruction of the tissue that the results could not be estimated The stainings for proteins (Table 1) were rather insignificant but faint reactions were seen in both the vacuolated cells of the eyes and in the cytoplasm of distended ganglion cells

Fig 4 Spatia of the sclera probably *fibrocytes with* granular material (arrows) Formalin fixation Frozen section Haematoxylin eosin Lab no 344 68 (× 275)

Fig 5 Spatia of the cornea *mainly empty* with remaining nuclei Same procedure as in Fig 4 Lab no 344/68 (× 275)

Fig 6 Ciliary body and processes with *numerous* distended cells The stored *material has disappeared* Formalin fixation Paraffin embedding Van Gieson Lab *no 343/68* (× 275)

Fig 7 Ballooning *of the Purkinje* cells of the cerebellum *Note the granular* cytoplasm Formalin fixation Gelatin embedding Frozen section Haematoxylin eosin Lab no 44/70 k (× 275)

2 Merck Darmstadt no 7192
3 Fluka AG Switzerland no 403019
4 Behringswerke Marburg Lahn no 20261


6
7

TABLE 1 *Staining Reactions of the Stored Material*

Substance	Method	Significance	Eye	Brain
Lipids	Birefringence	Cholesterol esters Lipins }	—	—
	Oil red O	Lipid material	—	(+)
	Sudan black B	Lipid material	—	(+)
	Luxol fast blue	Gangliosides Cerebrosides Phospholipids }	—	++
	Phosphomolybdic acid	Choline—containing lipids	—	(+)
	Nile blue (Cain)	*Neutral lipids*	—	—
		Acidic lipids	—	++
	Cresyl violet-acetic acid (2)	Sulphatides	—	—
Proteins	Mercury—bromphenol blue (40)	Proteins	—	—
	Oxidized tannin azo (OTA)	Tannophilic protein	(+)	(+)
	Chloramine-T Schiff	Protein bound NH_2	(+)	(+)
	Ninhydrin Schiff	Protein bound-NH_2		(+)
	Mixed anhydride	Protein bound COOH	—	—
	Alkaline tetrazolium	-SS- and -SH- groups	(+)	(+)
	Dihydroxy dinaphthyl disulphide (DDD)	-SH groups	—	
	Performic acid-alcian blue	-SS- groups	—	
	Diazotization coupling	Tyrosine		—
	DMAB nitrite	Tryptophan	—	—
	Gallocyanin chromalum (19)	RNA	—	(+)
Polysaccharides	Best's carmine (39)	Glycogen		—
	Periodic acid Schiff (PAS) (44, 45)	Vicinal glycols	—	+++
	0,02 per cent Azure A (AA) pH 1,5	Metachromasia or Orthochromasia }	—	—
	0,02 per cent Azure A pH 2,0		—	—
	0,02 per cent Azure A pH 3,0		(+)M	(+)O
	0,02 per cent Azure A pH 4,0		+M	+0
	Alcoholic toluidine blue (TBL)*		+M	+0
	Aqueous AA		+M	+0
	Wet 0,5 per cent aqueous AA			+0
	Astrablau pH 1,0	Sulphated MS	(+)	+
	Alcian blue (AB) pH 1,0 (65)	Sulphated MS	+	+
	Alcian blue pH 2,5 (65)	Acidic MS	++	++
	AB CEC 0,1 M $MgCl_2$ (63)	Differentiation of acidic MS }	+++	+++
	0,2 M $MgCl_2$		+++	+
	0,5 M $MgCl_2$		+++	—
	0,8 M $MgCl_2$		++	—
	1,0 M $MgCl_2$		++	—
	Resorcinol nitrite (2)	Sialomucins *Gangliosides* }		(+)
	Colloidal iron (CI)	Acid MS	+++	
	Safranin O (37)	Strongly acidic MS	(+)	(+)
	Aldehyde fuchsin (AF) (51)	Sulphated MS	+	
	CI PAS	Differentiation of acid and neutral MS	+++§ —	
	AF AB	Differentiation of sulphated and other acidic MS }	+§ ++	
	High iron diamine AB		+§ ++	

TABLE 1 (*Cont*)

..bstance	Method	Significance	Eye	Brain
arious	Haematoxylin eosin		(+)B	+B
	Van Gieson		Y	
	Sirius		Y	•
	Perls reaction	iron	—	—

* Toluidine blue O Merck Darmstadt no 1273

	= Staining not performed or unsuccessful
—	= No staining
(+) → + + +	= Degree of staining
M	= Metachromasia
O	= Orthochromasia
B	= Basophilia
Y	= Yellow
MS	= Mucosubstances

§ Upper row Reactivity of the first dye in the sequence
Lower row Reactivity of the second dye in the sequence
Numbers in parentheses refer to the list of references

No glycogen could be demonstrated by *Best s* carmine or by diastase digestion fol lowed by PAS This reaction was negative in *the spatia of the* eyes *but heavy* in the gang lion cells (Fig 10) Phenylhydrazine blockade (PAPS) (Table 2) did not change the PAS-reaction, which was blocked in all the sur rounding tissue (Fig 11) The cytoplasm of the ganglion cells was orthochromatic, while metachromasia could be demonstrated in the eyes neither at a low pH, nor by oxidative deamination (Table 2), but only at or above pH 3 0 (Fig 15)

The Astrablau and Alcian blue (AB) stainings at pH 1 were slightly positive in both tissues and at pH 2,5 were heavy in

TABLE 2 *Staining of Stored Material after Blockade and Extraction*

Substance	Method	Significance	Eye	Brain
	Phenylhydrazine blockade + PAS (PAPS) (37)	Differentiation of neutral MS and Sialomucins	—	+ + +
	Oxidative deamination (3) + AA pH 0 5	Prevention of competitive effect of proteins on meta chromatic staining	—	—
	AA pH 1 5		—	—
Polysaccharides and Lipids	Sulphation (36)	Induced metachromasia		(+)M
	Dimedone blockade (16h) + PAS (5)			+ +
				(+)
				—
				—
				+ + +
				+
				+ +

Symbols

TABLE 1 *Staining Reactions of the Stored Material*

bstance	Method	Significance	Eye	Bra·
	Birefringence	Cholesterol esters Lipins	—	—
	Oil red O	Lipid material	—	(+
	Sudan black B	Lipid material	—	(+
ıids	Luxol fast blue	Gangliosides Cerebrosides Phospholipids	—	+ +
	Phosphomolybdic acid	Choline—containing lipids	—	(+
	Nile blue (Cain)	Neutral lipids	—	—
		Acidic lipids	—	+ +
	Cresyl violet acetic acid (2)	Sulphatides	—	—
	Mercury—bromphenol blue (40)	Proteins	—	—
	Oxidized tannin azo (OTA)	Tannophilic protein	(+)	(+)
	Chloramine T Schiff	Protein bound NH_2	(+)	(+)
	Ninhydrin Schiff	Protein bound NH_2		(+)
	Mixed anhydride	Protein bound COOH	—	—
ıteins	Alkaline tetrazolium	SS and SH groups	(+)	(+)
	Dihydroxy dinaphthyl disulphide (DDD)	*SH* groups	—	
	Performic acid alcian blue	SS groups	—	
	Diazotization coupling	Tyrosine		—
	DMAB nitrite	Tryptophan	—	—
	Gallocyanin chromalum (19)	RNA	—	(+)
	Best s carmine (39)	Glycogen		—
	Periodic acid Schiff (PAS) (44 45)	Vicinal glycols	—	+ + +
	0 02 per cent Azure A (AA) pH 1 5	Metachromasia or Orthochromasia	—	—
	0 02 per cent Azure A pH 2 0		—	—
	0 02 per cent Azure A pH 3 0		(+)M	(+)
	0 02 per cent Azure A pH 4 0		+M	+O
	Alcoholic toluidine blue (TBL)*		+M	+O
	Aqueous AA		+M	+O
	Wet 0 5 per cent aqueous AA			+O
	Astrablau *pH 1 0*	*Sulphated MS*	(+)	+
	Alcian blue (AB) pH 1 0 (65)	Sulphated MS	+	+
	Alcian blue pH 2 5 (65)	Acidic MS	+ +	+ +
	AB CEC 0 1 M $MgCl_2$ (63)	Differentiation of acidic MS	+ + +	+ + +
	0 2 M $MgCl_2$		+ + +	+
ysaccharides	0 5 M $MgCl_2$		+ + +	—
	0 8 M $MgCl_2$		+ +	—
	1 0 M $MgCl_2$		+ +	—
	Resorcinol nitrite (2)	Sialomucins Gangliosides		
	Colloidal iron (CI)	Acid MS	+ + +	(+)
	Safranin O (37)	Strongly acidic MS	(+)	(+)
	Aldehyde fuchsin (AF) (51)	Sulphated MS	+	
	CI PAS	Differentiation of acid and neutral MS	+ + +§ —	
	AF AB	Differentiation of sulphated and other acidic MS	+§ + +	
	High iron diamine AB		+§ + +	

TABLE 3. *Classification of Mucopolysaccharidoses*
(Modified after V. McKusick (41))

Type	Name	Signs and symptoms	Mode of inheritance	Urinary excretion
I	Pfaundler Hurler	Grave gargoyle features Early corneal opacity Mental retardation	Autosomal recessive	Chondroitin sulphate B Heparitin sulphate
II	Hunter	Mild gargoyle features. Adults No corneal opacity Mild mental impairment	Sex linked recessive	Chondroitin sulphate B Heparitin sulphate
III	Sanfilippo	Mild gargoyle features Corneal opacity? Early mental retardation	Autosomal recessive	Heparitin sulphate
IV	Morquio	Grave skeletal symptoms No corneal opacity No mental retardation	Autosomal recessive	Keratosulphate Chondroitin sulphate B
V	Scheie	Mild skeletal symptoms. Adults Severe corneal opacities Mild mental disturbance	Autosomal recessive	Chondroitin sulphate B
VI	Maroteaux Lamy	Grave skeletal symptoms Corneal opacities Normal intellect	Autosomal recessive	Chondroitin sulphate B
VII	Dyggve Clausen Melchior	Specific skeletal symptoms Corneal opacities varying Mental retardation varying	Autosomal recessive	Chondroitin sulphate B Keratosulphate Hyaluronic acid
VIII	Durand van Hoof	Progressive spasticity No corneal opacity Mental retardation	Autosomal recessive	Fucose rich MPS

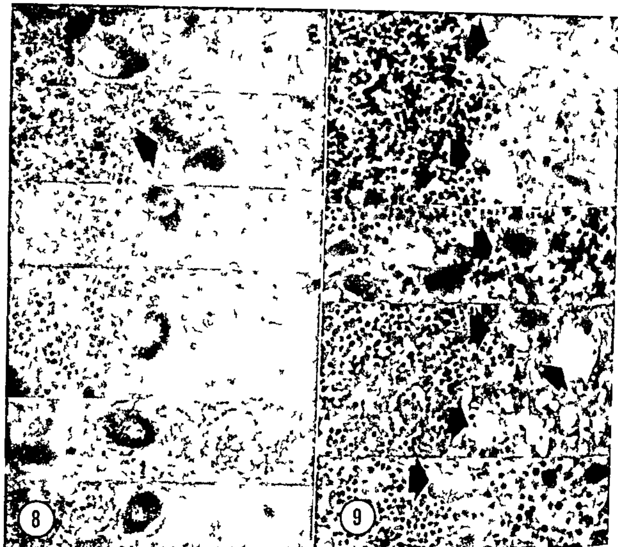


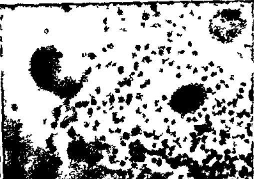
Fig 8 Copper phtl alocyanin positive material in d stended Purkinje cells Tv o cells are part cula ly swollen (arrows) Formalin f xat on Gelat n embedd ng Frozen sect on Luxol fast blue MBS Lab no 44/70 i (× 275)

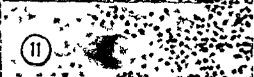
Fig 9 Cold pyridine extract on follo ved by PAS Tl e stored material extracted to a large extent lea ing a fa ntly sta ned cytoplasm (arro vs) Compare w th F g 10 Same techn que as in Fig 8 Lab no 44/70 j (× 275)

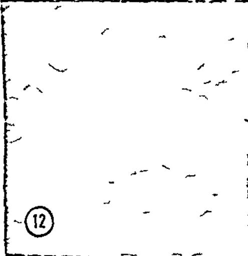
both The critical electrolyte concentration method in conjunction with AB staining was highly significant as it showed total negative staining in the brain at a concentration of MgCl above 0 2 M but the staining was still significant in the eye at a molar concen tration of 1 0 (Fig 12 13) Tl e granular material in the ocular spatia was Hale pos itive (Fig 14) and Perl negative while the Safranin O the high iron diamine (HID) stainings and the latter combined with AB gave only faint reactions indicating insignif icant amounts of strongly acidic/sulphated MS Aldel yde fuchsin according to *Prento* (51) showed undoubtedly a positive staining of the granules mainly in spatia in the inner layers of the sclera indicating the presence of sulpl ate groups The resorcinol nitrite method for sialic acid and gangl oside d d not work in our hands but this method prob ably demands cryostat sections of unfixed tissue thereafter fixed in formaldel yde vapo ir

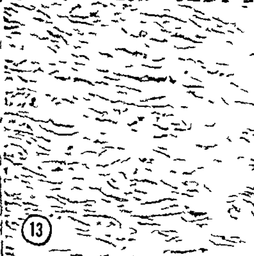
Regarding paraffin sections of brain tissue















18

from the patient, both the PAS reaction and the Alcian blue stainings were negative In a case of cardiac arrest in a 6 year old boy in connection with severe chest and abdominal trauma, *Klinken* (32) found PAS positive cytoplasm of ganglion cells in paraffin sections This finding was confirmed by me in examining paraffin sections of tissue from the same brain The PAPS reaction was however, negative on this material The PAS reaction on paraffin sections of cerebellum of the present case was negative

DISCUSSION

The following items will be discussed

A The clinical diagnosis
B Some histochemical problems
C The present findings compared with previous findings, especially the ocular findings
D Comparison of the histochemistry with the probable biochemical defect

A The former term, dysostosis multiplex or gargoylism (now mainly used of Pfaundler-Hurler's syndrome), is at present split up into at least eight types of mucopolysaccharidoses, although this distinction is not always clear cut or easy A survey of these types may be seen in Table 3 Although the initial diagnosis of the present case was Hurler's syndrome or atypical Hurler's syndrome, a revision of the clinical and laboratory data by the pediatricians (42) led to the conclusion that Sanfilippo's syndrome was the most probable Hunter's syndrome was much less probable and Hurler's syndrome excluded The delayed severe mental retardation in the boy together with the very mild gargoyle features the mild skeletal changes with no gibbus and clear corneas were strong support for this diagnosis Only in one case of Sanfilippo's syndrome have mild corneal opacities been observed (22)

The examination of the excreted AMP in the urine was by the method applied unfortunately of no use in distinguishing exclusively heparitin sulphate, which is mainly excreted in Sanfilippo's syndrome, from a mixture of chondroitin sulphate B and heparitin sulphate, which are excreted particularly in Hurler's syndrome (11) (Discussion of the problems of separation of these AMP, see 57, 68)

B The stored material in mucopolysaccharidoses, mainly stressed in works on Hurler's disease, is fairly easily soluble in aqueous solutions (10, 13, 30, 31, 59, 75) It is found best preserved in absolute alcohol Carnoy's solution Bouin's solution, lead acetate and some others (59) *Klika & Klouček* (31) however, found the material preserved in formalin fixed tissue, as was the case in the present material, although a rather large part of it undoubtedly had become dissolved and had disappeared giving the vacuolar pattern in the cornea and sclera so often seen in the early works on gargoylism But as clearly seen in the CI PAS stain (Fig 14), a significant amount was preserved in the ocular tissues of the present case On the other hand one must bear in mind this easy solubility when assessing the sections especially after enzyme digestion Control sections are mandatory, partic

Fig 10 PAS positive swollen Purkinje cells (arrows) Same technique as in Fig 8 Lab no 44/70 1 (× 275)

... followed by PAS
... anged in the
... he surround

Fig 12 The Alcian positive granular material in the scleral spaces and the alcianophilic connective tissue Formalin fixation Frozen section AB CEC procedure at an ion concentration of 0 1 M $MgCl_2$ Lab no 343/68 (× 275)

Fig 13 Same as Fig 12 at an ion concentration of 1 M $MgCl_2$ Note the preserved alcianophilia of the spaces Lab no 343/68 (× 275)

Fig 14 The Hale positive material of the scleral fibrocytes Formalin fixation Frozen section Colloidal iron—PAS after Mowry Lab no 343/68 (× 275)

Fig 15 Metachromasia at pH 3 0 of the material in scleral fibrocytes Formalin fixation Frozen section Toluidine blue O Lab no 344/68 (× 275)

(Fig 15), but not below, as should have been expected if sulphated MS were present On the other hand, Astrablau and AB did stain at pH 1 0 and what I personally find of importance is the preserved AB staining at an ion concentration of 1,0 M (Fig 13) This is consistent with the presence of sulphated MS (63), as is also the positive AF staining according to *Prento* (51) In this respect it may be of interest that heparitin [illegible]

patients showed synthesis of both sulphated and non sulphated AMP (58) The grade of sulphation had also previously been found to vary in gargoylism (69) In the first published case of *Sanfilippo*'s syndrome (26, 56) it was particularly noted that the typical intense metachromasia of Hurler's syndrome seen in liver cells was not observed, but only minimal metachromasia with TBL, and *Wallace et al* (72) found no metachromasia, but CI positive vacuoles in liver cells

The resistance to hyaluronidase is common to chondroitin sulphate B and heparitin sulphate (71) and a differentiation is therefore impossible on an enzymatic basis

In Table 4 it can be seen that sulphated MPS in the brain tissue are indicated by an (+) This was done because a positive AB stain at pH 1 0 was present But on the other hand the AB staining using the AB CEC method is abruptly reduced at a molarity of 0 2 M MgCl which is characteristic of carboxyl groups as in sialic acid containing substances This fact together with a significant reduction of AB staining after pyridin extraction probably indicates that gangliosides are prevalent in the distended ganglion cells

Summarizing the findings the predominant accumulated material in the ganglion cells of the brain is probably of a ganglioside nature Small amounts of MPS and glycoproteins may also be present In the eyes the granular material is consistent with acidic MS which are hyaluronidase and neuraminidase resistant and probably sulphated Because of the nature of the metachromatic reactions, it is suggested that a large content of heparitin sulphate with its somewhat few sulphate groups is present, supporting the clinical diagnosis of Sanfilippo's syndrome

C Histochemical examination of biopsy and necropsy material of mucopolysaccharidoses, mainly Hurler's syndrome, has not yielded consistent results This may be due in part to the various methods of fixation (see above) and in part to examination of various types of mucopolysaccharidosis, particularly in the early-examined cases where a differentiation of the various types was not possible, most cases being described as *gargoylism* but probably being mainly cases of Hurler's syndrome The histochemical methods applied, yielding varying results have given evidence of the disease being a lipidosis, a glycogenosis, a mucoproteinosis and a mucopolysaccharidosis (9, 10, 12, 38, 41, 60, 61, 64, 68, 75)

The PAS-reaction has always been applied in these investigations Our study of anoxic brains however, shows that a PAS reaction *without* further measures is insignificant when studying brain tissue of cases of mucopolysaccharidosis

Since the first case of Sanfilippo's syndrome was described in 1961 (26) about 60 cases have been published (53)

The basic histopathological finding, the cytoplasmic swelling of parenchymal and ganglion cells as found in the present case and in other cases of the same syndrome (19, 68), is similar to the findings in other types of mucopolysaccharidosis (9, 10, 12, 23, 27, 33, 38 67)

In the rather few cases of Sanfilippo's syndrome where histochemistry has been carried out (7, 25 57, 68, 72), the results have also been inconsistent *Sanfilippo et al* (56, 57) found in one case metachromasia in the liver cells and in another none *Wallace et al* (72) found lipid in neurons and metachromatic material in macrophages of the brain but no metachromasia in the liver The vacuolated liver cells, however, were Hale positive

TABLE 4 *Summary of Results*

Substance	Eye	Brain
Protein	(+)	(+)
Fat	—	(+)
Glycolipids	—	+
Glycogen	—	—
Neutral MPS	—	+
Acidic MPS	+	+
sulphated	+	(+)
hyaluronidase resistant	+	+
sialic acid containing	—	+
Mucoproteins Glycoproteins	—	(+)

(+) Small amounts or doubtful presence
+ Significant presence
MPS Mucopolysaccharides

ularly here When studying cerebral tissues where changes in the lipids are suspected fixation *in alcoholic solutions has to be* dissuaded

Judged from the histochemical reactions the stored material in the eyes and brain consisted of the groups of substances seen in Table 4 The difference in eye and brain is evident The eye contains no lipid at all while the material in the ganglion cells is apparently rich in glycolipids probably gangliosides This is estimated from the facts that dimedone blockade does not affect the PAS reaction of the ganglion cells indicating a glycolipid (5) and that the PAS reaction is strongly affected by pyridin extraction and unaffected by ether extraction which should be characteristic of protein bound gangliosides and cerebrosides in formalin fixed tissues (17) Free gangliosides are very soluble and do not stain in tissues (1) A histochemical differentiation by a single method between cerebrosides and gangliosides is to the best of my knowledge not possible It is also characteristic that glycolipids are only faintly stained by red Sudan dyes (1) and that Sudan black B is far more soluble in phospholipids than in glycolipids (49) consistent with *the faint fat stainings in the* present brain *material* The positive Luxol fast blue is therefore probably due to gangliosides or cerebrosides and not to phospholipids This is further supported by the orthochromasia of the ganglion cells as phosphatides *are probably* the principal lipid substances inducing metachromasia of lipid material (49) The blue colour by the Nile blue sulphate method of *Cain* on the other hand indicates acidic lipids and gangliosides react acidically *due to one or more* N acetyl neuraminic acid residues (66) It is probable that the positive PAPS reaction indicating sialic acid (65) is due to those sialic acid residues of gangliosides and not to sialomucins as *indicated in Table 4, but exact* histochemical differentiation is not possible The fact that neuraminidase did not change the PAS reaction or the AB staining may seem contradictory, but may perhaps be simply explained by these small residues of sialic acid in gangliosides not abundant *enough to give a significant reduction by* neuraminidase and thereby strengthening the indication of gangliosides in the stored material On the other hand both slowly digested and neuraminidase stable sialomucins exist (49) *It is probable that the N acetyl* neuraminic acid (NANA) which is in 2 3 *glycoside linkage to the internal galactose is* relatively more resistant to hydrolysis by neuraminidase than other NANA residues (62) The 2–3 linkage is found mainly *in the* sialogangliosides biochemically increased in gargoylism *and variants (62) Very* small amounts of protein, assessed on the protein stainings were found in *the brain and it* cannot be ruled out that the remaining PAS reaction after lipid *extraction is due to glyco*proteins or mucoproteins together with other neutral MS No significant reductions by *pepsin however could* be found The protein is probably bound to gangliosides (see above) In the eyes the granular *material gave only* reactions for acidic MPS *together* with a few faint reactions *for protein Pepsin* digestion did not change the staining reactions and *particularly no* increase of the stainings were *found* as would have been expected if anion groups bound to protein had been liberated *by the proteolytic enzyme*

A point of special interest and also a little confusing is *the metachromasia at pH 30*

Melchior et al (43) found in a case of Hurler's syndrome an increase and change in the gangliosides and an increase of AMP in organs other than the brain In a later study, *Clausen et al* (12) found an increased level of mono- and di sialogangliosides in the brain matter and found it reasonable to explain the findings in gargoylism by a lack of a β galactosaminidase-like enzyme, leading to accumulation of glycolipids chondroitin sulphate and their precursors They suggested an interrelationship between mucopolysaccharidoses and lipidoses and found that both could be explained by a lack of degradative enzymes due to abnormalities in the lysosomes

The distended lysosomes should then appear as the vacuoles in light microscopy (6, 7 25, 29, 34, 68) Both the endoplasmic reticulum (31) and the Golgi apparatus (14), however, have been suggested as origins of the vacuoles

The biochemical changes therefore appear to be quite well reflected in the histopathological and histochemical findings of this case I find it more probable that a basic enzyme defect in the lysosomes leads to an accumulation of the granular material than that material coming from outside the cell is deposited The accumulation in fibrocytes of connective tissue, parenchymal cells and ganglion cells with their different normal cytoplasmic constituents leads probably to different histochemical findings especially in cerebral tissue and connective tissue

It is reasonable to believe that the pathogenesis resulting in a uniform histological morphology is the same for all types of mucopolysaccharidosis, but that variations in the defective enzymes cause the different types

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A thorough histochemical investigation by *Feller et al* (68) and *Bechtelsheimer et al* (7) of two biochemically established cases of Sanfilippo's syndrome showed in liver cells stored AMP *which were only faintly meta-*chromatic but PAS-positive, which led to the conclusion that neutral MPS were probably also present In addition, a proteinaceous material was suggested, while lipids were ex-*cluded Histochemical examination of cer-*ebral tissue in two cases of Sanfilippo's syndrome revealed a neuronal overload, probably of gangliosides (19, 25) The ultrastructure of cells in Sanfilippo's syndrome has *been found to be similar to that of Hurler's* syndrome (7, 19, 25, 68, 72)

Histopathological examination of the eye has been carried out in mucopolysaccharidoses nearly exclusively in cases of Hurler's *syndrome, in part as a study of the cornea* (4, 8, 15, 21, 31, 46, 55, 70, 73) and in part as a study of whole eyes (27, 28, 33, 38, 48, 54, 74, 75, 76) In the latter studies corneal changes were found exclusively, apart *from six cases in four of which (27, 48, 74,* 75) scleral changes similar to the scleral changes of the present case were found, although not so prominent In one case (38) vacuolar changes were found in the iris, *ciliary body and choroid, and in one in the* retina (4) morphologically similar to the changes found in the ciliary processes of the present case

In several of the cases mentioned above, a number of histochemical methods have been applied, mainly stainings for lipids In general, fat stains and a variety of others including stains for polysaccharides *have usually* been negative Mucopolysaccharides appear to have been demonstrated in only a few (21, 31, 75) Electron microscopy of the cornea in Hurler s syndrome has so far been carried out *in four cases (15, 31, 50, 55)* showing spheres or membrane bound bodies with a granular matrix or lamellar inclusions as also shown in fibroblast cultures *(6)*, skin fibro blasts (14) and liver cells (29)

A *whole* eye has been studied in Hunter s syndrome (23), and as in the present case scleral and ciliary body changes were found in addition to minimal stromal depositions of the cornea This case is claimed to be the first with clear corneas to be studied histopathologically

The conjunctiva and the cornea in Scheie s syndrome have been thoroughly investigated histochemically (59), and deposits of AMP rendered probable, although the demonstrat*ed metachromasia of the vacuolated cyto*plasm is not seen to have been demonstrated at a low pH

Histopathological and histochemical examination of the cornea has been studied in *one case of Morquio's syndrome (47)* The eyes in a case of Maroteaux Lamy syndrome have recently been studied (24) Deposition of MPS in cornea and sclera were found

The present case of Sanfilippo's syndrome *presents the same basic ocular changes as in* other types of mucopolysaccharidosis, but with the main changes in the sclera and only few changes of the cornea Compared with the published cases scleral and ciliary body *changes are rare and the present* findings further show that corneal changes histo pathologically may be found even if the corneas are clear clinically Probably the presence of large storage cells immediately beneath the epithelium is necessary for the clinical appearance of cloudiness of the cornea

D Brante (10) was the first to recognize gargoylism as a mucopolysaccharidosis and to emphazise the difference in properties of the substances in nerve cells and other tissue This difference has been pointed out a num*ber of times since then* (9, 13, 30, 48, 69 75) and a gene regulated disturbance of the sphingoglycolipid and polysaccharide metabolism has been assumed (61) *Dorfman* (16) suggested that the metabolic defect in Hurler's syndrome may reside in a defect of the peptide or linkage of the peptide to poly saccharide resulting in failure of the acid mucopolysaccharide to be *fixed normally* in connective tissue, and subsequently by the blood stream to become deposited in cells and excreted in the urine

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In addition to registering Mallory bodies (3), and alcoholic hepatitis (4), estimation of the biopsi as an entity has been performed with a classification of the material according to chief histological diagnoses

RESULTS

Table 1 shows the distribution according to chief histological diagnosis of the 287 biopsies comprising the material and in addition the average age of the patients in the individual groups

279 of the biopsies (97 per cent) are from men in the age group 23–78 years (average age 50 years) Eight biopsies are from women in the age group 28–52 years (average age 44 years)

Cirrhosis

38 of the biopsies (13 per cent) fulfill the histological criteria for cirrhosis (nodular regeneration and fibrosis) 37 of the 38 biopsies with cirrhosis are from men and the average age for the whole group is 54 years All the biopsies with the exception of two show some degree of fatty change

TABLE 1 *Distribution of the 287 Biopsies of the Material According to Chief Histological Diagnosis and Average Age of the Individual Groups*

Chief histological diagnosis		Number of biopsies	Average age
Cirrhosis with	slight fatty change	9	54
	moderate fatty change	25	54
	severe fatty change	2	45
Cirrhosis without fatty change		2	59
Obs cirrhosis with fatty change		5	53
Fatty liver	slight degree	95	52
	moderate degree	69	50
	severe degree	24	46
Haemosiderosis		23	49
Viral hepatitis		3	34
Chronic aggressive hepatitis		2	50
Liver tissue with granulomas		1	48
Liver with non specific changes		2	46
Liver with pathological changes		24	46
Unsuitable biopsy		1	39
		287	

One of the biopsies with cirrhosis but no fatty change shows bile ducts with atypical epithelium (9) and in addition a dense infiltration of the connective tissue in the septa with lymphocytes and plasma cells, and one of the biopsies with cirrhosis and fatty change in addition shows hepatoma

In five biopsies (two per cent), all with fatty change, the histological picture have given a suspicion of cirrhosis but have not been diagnostic

Fatty Liver

Somewhat more than half (66 per cent) of the biopsies show fatty infiltration without cirrhosis Five of the biopsies are from women (average age 40 years) The average age for men with severe fatty infiltration is lower (46 years) than the average age of the other groups with fatty infiltration, and also lower than the average age for the whole group with cirrhosis

Haemosiderosis

Eight per cent (23 biopsies) show some degree of haemosiderosis without simultaneous fatty change and/or cirrhosis One of the biopsies is from a woman, and the average age for the whole group is 49 years

Others

The groups comprising biopsies with changes compatible with viral hepatitis (2), chronic aggressive hepatitis (2), granulomas (1) and non specific reactive changes (2) are all small, and the distribution stated according to age may reflect an incidental variation All the patients in these groups are males 24 biopsies (eight per cent of the material) show normal liver tissue, and only one biopsy contained insufficent liver tissue for a morphological evaluation

Mallory Bodies

Mallory bodies have been found in 29 biopsies (ten per cent) All biopsies with

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THE FREQUENCY OF MALLORY BODIES IN LIVER BIOPSIES FROM CHRONIC ALCOHOLICS

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In a material consisting of liver biopsies from 287 consecutive chronic alcoholics Mallory bodies have been found in 29 Of these 18 show cirrhosis and fatty infiltration (corresponding to 44 per cent of the total number of specimens showing cirrhosis and fatty change) and 11 (corresponding to six per cent) show fatty change but no cirrhosis, while Mallory bodies have not been demonstrated in biopsies without fatty change The incidence of Mallory bodies and alcoholic hepatitis in consecutive materials of liver biopsies from chronic alcoholics appears to be ten and eight cent respectively

Most materials dealing with the incidence of Mallory bodies have been selected both on morphological and clinical criteria Thus *Baggenstoss & Stauffer* (1) *Hall & Morgan* (6), and *Popper & Szanto* (8) found that alcoholic cirrhosis with fatty infiltration contained Mallory bodies in 40–93 per cent, and Popper & Szanto (8) demonstrated Mallory bodies very frequently in alcoholics with fatty liver

In a previous paper one of the authors (3) has given the incidence and frequency of Mallory bodies in 1,100 consecutive liver biopsies from a Scandinavian metropolis In 62 (six per cent) of these 1,100 biopsies Mallory bodies have been demonstrated

To the knowledge of the authors no calculation of the frequency of Mallory bodies in a larger consecutive material of chronic alcoholics exist

Received 10 xi 70

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The main objective of this work has been to reflect with what frequency Mallory bodies are encountered in a consecutive series of liver biopsies from chronic alcoholics from a Scandinavian metropolis

MATERIAL AND METHODS

The material consists of 287 percutaneous liver biopsies from 287 consecutive chronic alcoholics admitted to department II, Kommunehospitalet

The biopsies have been received at the Pathological department, Kommunehospitalet during the period January 1968 to October 1970

The liver biopsies have been performed when there was a history of alcohol consumption of more than 50 g/day for a period of greater duration than a year All the patients in department II and their nearest relatives have in the investigation period been specifically questioned on this point by one of the authors (K N) The biopsies are 1–1 4 mm thick and 1 5–4 5 cm long and are cut in serial sections (6 μm 50 sections)

The assessment has been performed on haematoxylin and eosin and v Gieson Hansen stained sections Furthermore, additional sections from all biopsies stained for reticulin fibres (5) iron (7) and pyroninophil substance (2) have been available

In addition to registering Mallory bodies (3), and alcoholic hepatitis (4) estimation of the biopsy as an entity has been performed with a classification of the material according to chief histological diagnoses

RESULTS

Table 1 shows the distribution according to chief histological diagnosis of the 287 biopsies comprising the material and in addition the average age of the patients in the individual groups

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	moderate degree	69	50
	severe degree	24	46
Haemosiderosis		23	49
Viral hepatitis		3	34
Chronic aggressive hepatitis		2	50
Liver tissue with granulomas		1	48
Liver with non specific changes		2	46
Liver with pathological changes		24	46
Unsuitable biopsy		1	39
		287	

age for the whole group is 54 years All the biopsies with the exception of two show some degree of fatty change

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Mallory Bodies

Mallory bodies have been found in 29 biopsies (ten per cent) All biopsies with

TABLE 2 Distribution of the 29 Biopsies in the Material Containing Mallory Bodies According to Histological Diagnosis and Average Age of the Individual Groups

Chief histological diagnosis		Number of biopsies with Mallory bodies	Average age
Cirrhosis with	slight fatty change	2(2)	54
	moderate fatty change	13(11)	55
	severe fatty change	1(1)	60
Suspicion of cirrhosis with fatty change		2(1)	59
Fatty liver	slight degree	–	
	moderate degree	6(5)	51
	severe degree	5(4)	47
		29	

( ) number of biopsies with alcoholic hepatitis in each subgroup

Mallory bodies are from men and all show fatty infiltration with or without cirrhosis Table 2 shows the distribution of the 29 biopsies according to chief histological diagnosis as well as the average age of the individual groups and the number of biopsies with alcoholic hepatitis

Mallory Bodies and Cirrhosis

Of the 29 biopsies with Mallory bodies 18 (62 per cent) belong to the groups cirrhosis with fatty infiltration and suspicion of cirrhosis with fatty change

In the entire material there are 41 biopsies showing cirrhosis (or suspicion of cirrhosis) with fatty change and Mallory bodies have thus been demonstrated in 44 per cent of these The average age for patients with cirrhosis fatty infiltration and Mallory bodies corresponds with the average age of the entire cirrhosis group Most biopsies with cirrhosis and Mallory bodies exhibit alcoholic hepatitis (83 per cent)

Mallory Bodies and Steatosis

Eleven biopsies with Mallory bodies belong to the group fatty infiltration without cirrhosis In the entire material there are 188 biopsies with fatty infiltration without cirrhosis, and Mallory bodies have thus been demonstrated in six per cent of these

The average age of patients with fatty change of the liver and Mallory bodies corresponds with the average ages of the entire groups with fatty change of the same degree (cf table 1 & 2) Alcoholic hepatitis is demonstrated in most biopsies with fatty change without cirrhosis but with Mallory bodies (82 per cent)

DISCUSSION

Frequency of Mallory Bodies and Alcoholic Hepatitis in Chronic Alcoholics

There is no information in the literature about the frequency of Mallory bodies in a material of biopsies from consecutive chronic alcoholics In a previous paper one of the authors (3) has given the frequency of Mallory bodies in a non selected series comprising 1100 consecutive liver biopsies to six per cent In the present material consisting of liver biopsies from 287 *consecutive chronic alcoholics* from the same Scandinavian metropolis Mallory bodies have been found in ten per cent In both materials Mallory bodies have only been demonstrated in biopsies showing fatty infiltration even though the fat and the Mallory bodies are not seen in the same cell

There is likewise no information as to how frequently alcoholic hepatitis (4) occurs in alcoholics In a previous paper *Christoffersen et al* (4) found alcoholic hepatitis in 45 (73 per cent) of 62 biopsies with Mallory bodies Most of the patients in this material (4) were clinically suspected of being chronic alcoholics and 84 per cent confirmed the diagnosis themselves (4)

In the present material 24 of 29 biopsies with Mallory bodies (82 per cent) concurrently exhibited alcoholic hepatitis (cf table

2) and thus alcoholic hepatitis has been found in eight per cent of the total material

Frequency of Mallory Bodies and Alcoholic Hepatitis in Chronic Alcoholics with Cirrhosis

In the part of the present material that comprises alcoholics with cirrhosis (or suspicion of cirrhosis) and fatty infiltration Mallory bodies have been demonstrated in 44 per cent *Rubin et al* (10) in an autopsy material of non consecutive cases of cirrhosis found that among alcoholics more than a third exhibited Mallory bodies *Baggenstoss & Stauffer* (1) and later *Popper & Szanto* (8) in non consecutive autopsy materials of alcoholics with cirrhosis demonstrated Mallory bodies in 72 and 93 per cent respectively

Christoffersen (3) demonstrated Mallory bodies in 45 (38 per cent) out of 120 consecutive biopsies with cirrhosis and fatty change No [illegible]

alcoholics and 84 per cent confirmed the diagnosis themselves (4) The figures for alcohol consumption are absolute minimum values since the information has been taken from case records and only part of the patients have been specifically questioned on this point.

The incidence of Mallory bodies in alcoholics with cirrhosis and fatty change thus appears to be 38–44 per cent in consecutive materials

The present material contains only two biopsies with cirrhosis without steatosis In none of these Mallory bodies are demonstrated

Alcoholic hepatitis is found in 83 per cent of the biopsies with cirrhosis and Mallory bodies

Frequency of Mallory Bodies and Alcoholic hepatitis in Chronic Alcoholics with Fatty Change but no Cirrhosis

There is no information in the literature as to how frequently Mallory bodies in consecutive materials of fatty livers from alcoholics *Popper & Szanto* (8) found Mallory bodies in 100 per cent of non consecutive cases of large fatty liver from alcoholics

Christoffersen (3) demonstrated Mallory bodies in 17 (seven per cent) of 253 consecutive biopsies with fatty change, but there is no information about the alcohol consumption

In the part of the present material that comprises fatty infiltration without cirrhosis Mallory bodies have been demonstrated in 11 (six per cens) of 188 biopsies It is noteworthy that Mallory bodies in the material of biopsies with fatty change from alcoholics are found with the same incidence as in the material of biopsies with fatty change without information regarding the alcohol consumption (3) This indicates that the patients with fatty change of the liver in the referred paper (3) to a large extent comprise alcoholics

It thus appears that the incidence of Mallory bodies in alcoholics with fatty change but no cirrhosis is about six *per cent* In patients with moderate to severe steatosis the incidence is somewhat higher approximately 12 per cent

The incidence of alcoholic hepatitis in alcoholics with steatosis and Mallory bodies but no cirrhosis is 82 per cent or just the same as in alcoholics with cirrhosis and Mallory bodies

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TABLE 2 Distribution of the 29 Biopsies in the Material Containing Mallory Bodies According to Histological Diagnosis and Average Age of the Individual Groups

Chief histological diagnosis		Number of biopsies with Mallory bodies	Average age
Cirrhosis with	slight fatty change	2(2)	54
	moderate fatty change	13(11)	55
	severe fatty change	1(1)	60
Suspicion of cirrhosis with fatty change		2(1)	59
Fatty liver	slight degree	–	
	moderate degree	6(5)	51
	severe degree	5(4)	47
		29	

( ) number of biopsies with alcoholic hepatitis in each subgroup

Mallory bodies are from men and all show fatty infiltration with or without cirrhosis Table 2 shows the distribution of the 29 biopsies according to chief histological diagnosis as well as the average age of the individual groups and the number of biopsies with alcoholic hepatitis

Mallory Bodies and Cirrhosis

Of the 29 biopsies with Mallory bodies 18 (62 per cent) belong to the groups cirrhosis with fatty infiltration and suspicion of cirrhosis with fatty change

In the entire material there are 41 biopsies showing cirrhosis (or suspicion of cirrhosis) with fatty change and Mallory bodies have thus been demonstrated in 44 per cent of these The average age for patients with cirrhosis fatty infiltration and Mallory bodies corresponds with the average age of the entire cirrhosis group Most biopsies with cirrhosis and Mallory bodies exhibit alcoholic hepatitis (83 per cent)

Mallory Bodies and Steatosis

Eleven biopsies with Mallory bodies belong to the group fatty infiltration without cirrhosis In the entire material there are 188 biopsies with fatty infiltration without cirrhosis, and Mallory bodies have thus been demonstrated in six per cent of these

The average age of patients with fatty change of the liver and Mallory bodies corresponds with the average ages of the entire groups with fatty change of the same degree (cf table 1 & 2) Alcoholic hepatitis is demonstrated in most biopsies with fatty change without cirrhosis but with Mallory bodies (82 per cent)

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In the present material 24 of 29 biopsies with Mallory bodies (82 per cent) concurrently exhibited alcoholic hepatitis (cf table

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CYTOMEGALY OF THE FOETAL ADRENAL CORTEX

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An analysis of the findings of adrenal cytomegaly in foetuses and infants in a five-year autopsy material showed that the frequency increased with gestational age and lifetime Therefore no basis was found for regarding cytomegaly as a normally occurring feature of foetal life In cases of erythroblastosis maternal toxaemia, and multiple pregnancies the frequency was found to be slightly increased, but the most striking feature was the high frequency of severe malformations It is discussed whether the high occurrence of malformations speaks in favour of viral induction or chromosome anomaly The cellular infiltration and the marked necrosis of the foetal cortex, which cannot be explained by the physiological involution, speak in favour of a viral aetiology Finally, the resemblance between cytomegaly and the cellular changes in idiopatic Addison's disease and congenital adrenal hypoplasia of cytomegalic type is mentioned

The occurrence of large atypical cells in the foetal adrenals from human foetuses and newborn infants was first described by *Craig & Landing* in 1951, and later in several greater materials of otherwise normal adrenals in this age group (3, 4, 7, 13, 25, 29)

There is some disagreement about the importance of these cells *Kampmeier* (1927) suggested that they are a normal feature in the early foetal life, while others assumed that they are a manifestation of cytotoxic injury (3 12, 17, 21, 23), or might have the ability of developing into neoplastic cells (12) *Garneau* (1957) regarded these cells as changes caused by virus

In this investigation the occurrence of cytomegaly in a five year autopsy material has been analysed and correlated to other morphological elements in the adrenals such as haemorrhage, necrosis and cellular infiltration to the findings in the other organs and to the clinical findings in the mother and the child

Received 28 x 70

MATERIAL AND METHODS

The material consists of 927 adrenals from foetuses at ages ranging from 12 weeks of gestation up to infants living for 11 years 25 cases showed the characteristic changes of cytomegaly Among these, two were stillborn infants with a length of feet of 35 and 62 mm, while the remainder were newborn living for periods covering from a few minutes up to seven days after delivery (Figure 1)

The adrenals were fixed in 10 per cent buffered formalin and stained with haematoxylin-eosin, van Gieson cresylviolet, methylviolet, and *Sudan Black* supplied with Sudan III, Oil red O, Scarlach Red and PAS on unfixed freeze sections The adrenals were studied in sections cut vertically through the hilus, and in a few cases in step sections In nine cases the adrenals on each side were examined

Cells with a size exceeding 20 my, with eosinofilic, often vacuolated cytoplasm, and hyperchromatic nuclei were indicated as cytomegalic cells (Figure 2) They were examined at a magnification × 35 The smallest number in one section was five cells The occurrence of cytomegaly was quantitated in four degrees Moreover, the foetal cortex was estimated with regard to occurrence of haemorrhage and necrosis according to the morphological criteria described earlier (5), and compared with a selected mate-

tive Study of Two Groups of Patients with Mallory Bodies with and without Liver Cell Necrosis and Neutrophilic Infiltration in Liver Biopsies Scand J Gastroent *5* 633–638, 1970

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TABLE 1 *Clinical Informations about the Pregnancy, Oestriol Excretion and Multiple Pregnancies of the 25 Cases of Adrenal Cytomegaly*

Autopsy number	The maternal conditions and treatments during pregnancy	The maternal urinary oestriol excretion	The multiple pregnancies
BS 80/68	Hydramnion		Trigemina A
BS 117/67	Toxaemia		–
BS 107/64	–	/	Trigeminus A
BS 208/65	Rh immunization	Decreased	–
BS 96/65	Treated with gestagens		–
BS 100/69	–		–
BS 238/66	–		–
BS 16/65	–		–
BS 122/67	–		Trigeminus C
BS 102/66	Rh immunization	Decreased	–
BS 154/66	Rh immunization		–
BS 28/66	Toxaemia	Decreased	–
BS 159/68	Treated with diuretica	Subnormal	–
BS 199/69	–		–
BS 30/65	–		Gemellus A
S 153/65	Epilepsia		–
BS 178/67	Rh immunization		–
BS 164/68	–		
BS 41/66	Toxaemia		
BS 247/65	Rh immunization		Gemellus A
BS 32/68	–		–
S 171/65	Treated with antibiotica		–
BS 176/69	Rh immunization		
BS 19/66	Toxaemia	Subnormal	–
S 141/65	–		–

except in one case, a girl of 1500 grs who lived for one day after delivery. During the pregnancy her mother suffered from severe toxaemia and had a low urinary oestriol excretion. The combined adrenal weight in this child was 1.95 grs (normally 3–4 grs) and represented 0.13 per cent (normally 0.3 per cent) of the body weight. Moreover, the foetal zone comprised only 64 per cent (normally 80 per cent) of the whole cortex, it showed a marked degree of cytomegaly and a massive necrosis of the foetal cortical cells.

In nine cases the adrenals on each side were examined, and in five of these cytomegaly was found in both.

Similar cellular changes were not found in the other organs. However, we found five cases with insular hyperplasia of the pancreas, two cases with hyperplasia of the Leydig cells, and one with ovotestis.

In nine cases the infants had severe malformations, five of these were multiple (Table 2).

Among the causes of death of the live born infants the hyaline membrane disease dominated with twelve cases, while eight had pulmonary haemorrhage, cerebral haemorrhage or pneumonia. In the remaining three cases we only found widespread atelectasis in the lungs.

The frequency of the cytomegaly in a five-year autopsy series was 2.7 per cent. The frequency was lowest among the stillborn

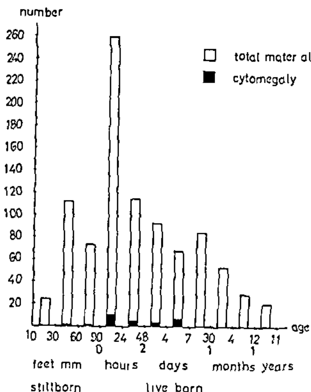


Fig 1 The distribution of the total material including the cases of cytomegaly in the different age groups

rial of 562 adrenals of foetuses and infants at ages ranging from 11 weeks of gestation to 2 years after delivery In this material all cases in which mothers presented with Rh immunization diabetes *mellitus*, *toxaemia* or decreased urinary oestriol excretion and cases of hydrops chromo

these criteria the material was regarded as normal

The width of the foetal zone was indicated in per cent of the total cortex and the weight of the *unfixed* adrenals was indicated as absolute and relative weight in relation to the body weight

In all cases the other organs were examined with regard to occurrence of similar cellular changes

The information about the mother the pregnancy and the delivery appear from Table 1 In five cases the maternal urinary oestriol excretion was determined

RESULTS

The cytomegalic cells were found either diffusely scattered or aggregated in foci in the foetal zone Around the foci and between the cells pronounced necrosis was frequently found The cytoplasm was positively stained with Sudan III, Scarlach Red Oil red O and PAS The nuclei were large and hyperchromatic containing several nucleoles and clumbs of chromatine, and sometimes inclusions of eosinofilic material We found no mitosis, but often cells with two nuclei The degree of cytomegaly was most marked in the adrenals of the older infants

In five cases a slight degree of cellular infiltration, composed of mainly lymphocytes was found to be located to the cytomegalic foci (Figure 3) All infants had lived for more than 24 hours after delivery The degree of necrosis in the adrenals with cytomegaly was more pronounced than in the corresponding groups in the normal material (Figure 4), but were not correlated to the degree of cytomegaly

The adrenal weight and the width of the *foetal zone* were within the normal range



Fig 2 Cytomegalic cells with large hyperchromatic nuclei and several nucleoles *Haematoxylin eosin* Magnification × 300

TABLE 2 *The Autopsy Findings in Nine Cases with Adrenal Cytomegaly and Malformations of Other Organs*

Autopsy number	The malformations
BS 96/65	– Truncus arteriosus communis, ventricular septal defect – Agenesia of oesophagus anus, gall bladder, kidneys, ureteres and bones of arms and legs – Hypospadia – Hydrocephalia with atresia of the aqueduct – Hypoplasia of the pituitary gland – Atresia of the external meatus
BS 159/68	– Diaphragmal hernia – Diffuse angiomatosis of the lungs and leptomeninges
BS 153/65	– Omphalocele
BS 164/68	– Ectopia of the right ureter ostium to vagina
S 171/65	– Atresia of oesophagus duodenum and anus – Hypospadia
BS 247/65	– Hypoplasia and ectopia of the left kidney
BS 32/68	– Atrial and ventricular septal defect
S 141/65	– Atresia of oesophagus and anus – Omphalocele – Recto-vaginal fistula Bicorn uterus
BS 199/69	Omphalocele Malrotatio of the intestine Ovotestis obs Stenosis of the aorta Macroglossia

an increased frequency of cytomegaly in cases of erythroblastosis (2 3 4 10, 12 25) We found six cases of cytomegaly among 40 infants with erythroblastosis

Driscoll et al (1960) and *Bayer et al* (1962) have indicated that the frequency of cytomegaly in the foetal adrenals may be increased in cases of maternal diabetes, and *Craig & Landing* (1951) demonstrated an increasing frequency in cases of maternal toxaemia The latter feature was also verified by our investigation but we did not find cytomegaly in our cases of maternal diabetes It was not verified either by *Oppenheimer* (1970)

19*

Several investigations have demonstrated an increased frequency of cytomegaly among infants with malformations (4, 13, 25, 28) In our material there were nine cases of malformations Three of these had omphalocele and one case could be classified as Beckwith s syndrome (BS 199/69, Table 2) in which bilateral adrenal cytomegaly is a characteristic feature This syndrome was first described by *Beckwith et al* (1964), but since then more than 16 cases have been published (8 11, 18, 19, 28) In a few of these cases an abnormal karyotype has been demonstrated (18 19)

Oppenheimer (1970) found an associa-



Fig 3 Cytomegalic focus with cellular infiltration Haematoxylin eosin Magnification × 140

infants (0 9 per cent), and highest among infants who lived from four to seven days after delivery (8 9 per cent) There were 12 boys and 13 girls Furthermore, five cases were gemelli or trigemini, constituting 6 per cent of the cases of cytomegaly, but only 2 7 per cent of the total material We examined the adrenals of the matching infants of the multiple pregnancies by step sections, but did not find corresponding cellular changes

Among the cases of cytomegaly we found six with erythroblastosis and four infants of mothers with toxaemia In the total material there were 40 cases of erythroblastosis and 27 infants of mothers with toxaemia Thus, the frequency of cytomegaly among these was 12 5 and 14 8 per cent respectively

In five cases the maternal urinary oestriol excretion was determined In three cases the terminal value was below the normal range, in two cases it was in the lower part of the normal range

DISCUSSION

The frequency of cytomegaly in the adrenals of stillborn and neonatally deceased infants is discussed, it was found to vary from one per cent (29) to 6 5 per cent (12) In a five-year autopy series comprising foetuses and infants at ages ranging from 12 weeks of gestation up to 11 years after delivery the frequency was found to range at 2 7 per cent However, the major part was neonatally deceased infants

In an autopsy material comprising 1039 post mortem examinations, *Craig & Landing* (1951) found 33 cases with cytomegaly The frequency was highest among the stillborn infants, while *Potter* (1961) found the frequency to be the same among the stillborn and the live born infants The frequency was found to increase with age of gestation and lifetime, but the oldest case was only seven days old, though the material comprised infants at ages up to 11 years

Kampmeier (1927), who studied the adrenals of five foetuses of varying ages of gestation, found cytomegalic cells in foetuses from the 8th to the 12th week of gestation, after this time the frequency decreased Therefore he suggested that these cellular changes were a normal feature in the embryonal life We did not examine foetuses younger than 12 weeks of gestation, but we studied 24 cases in which ages ranged from 12 to 15 weeks of gestation, and in none of these we found cytomegalic cells In 107 consecutive post mortem examinations of non-macerated stillborn infants, *Craig & Landing* (1951) found seven cases, the youngest being premature by 12 weeks

Among gemelli, cytomegaly has been found in both siblings (12, 29) In our material changes were found in only one of the siblings in cases of gemelli or trigemini, as shown by *Oppenheimer* (1970), but there was an accumulation of multiple pregnancies

Several investigations have demonstrated

the testis in one case, but regarded this as accessory adrenal tissue In our investigation, cells with two nuclei were often found, but they were exclusively located to the foetal zone and we did not find similar cellular changes in the other organs

Electron microscopic studies of the cytomegalic cells (8 28) revealed evidence of hyperactivity as numerous chromatin granula, intranuclear cytoplasmic inclusions, and markedly vesicular cytoplasm were present *Oppenheimer* (1970) studied the fine structure of a hyperplastic adrenal from a case of virilism, which showed similar patterns of hyperactivity indicative of active steroidogenesis

Some authors suggest that cytomegaly is caused by virus *Garneau* (1957) has published a case in which the cytomegaly of the adrenals was the only pathological finding at autopsy The mother of the child concerned had parotitis three weeks before the delivery On the basis of the resemblance with lesions experimentally produced by mumps virus he assumed that cytomegaly in this case had been caused by viral infection Yet electron microscopic investigations (8 28) have not demonstrated viral particles, and the nuclear inclusions, which resemble viral inclusions were demonstrated to be cytoplasmic invaginations (33) which have been observed in many types of cells under various conditions *Sobel et al* (1969) thought they were related to cellular hyperactivity However the high frequency of malformations speaks in favour of a viral aetiology

Dhom (1965) and *Kissane & Smith* (1969) have pointed out that cytomegaly in the adrenals resembles the cellular changes in idiopatic Addison s disease apart from the absence of cellular infiltration of lymphocytes and plasma cells We found a slight infiltration of lymphocytes, located to the cytomegalic foci in five cases and all the infants lived for more than one day As the physiological involution of the foetal adrenal cortex can be characterized as a non inflammatory haemorrhagic necrosis (5), this must be regarded as a pathological finding

Changes similar to cytomegaly are also seen in the congenital adrenal hypoplasia (21) All the published cases have been boys with clinical manifestations of adrenal insufficiency (1, 9, 15, 17, 23, 24, 26, 27, 32, 34, 35), and it is suggested that this disease is hereditary (9, 26, 34) *Oppenheimer* (1970) found one case of adrenal hypoplasia, but eight cases of hyperplasia among her 23 cases of cytomegaly In our investigation all the adrenals were of normal absolute and relative weight, except in one case, in which the foetal adrenal cortex was narrow with marked necrosis and cytomegaly The maternal urinary oestriol excretion in this case was abnormally low, which was the only indication of a decreased adrenal function in the child

In a case of adrenal hypoplasia of cytomegalic type, *Ehrlich et al* (1969) demonstrated a high plasma concentration of 20 alfa hydroxyprogesterone, which normally cannot be detected in human subjects They assumed that the cytomegalic cells had synthesized this agent which is a steroid of low biological activity, and pointed out that this agent might interfere with the normal steroid synthesis This might explain the occurrence of the cellular changes indicating hyperfunction in diseases characterized by adrenal insufficiency (15, 31), and also the finding of decreased maternal urinary oestriol excretion in cases of cytomegaly

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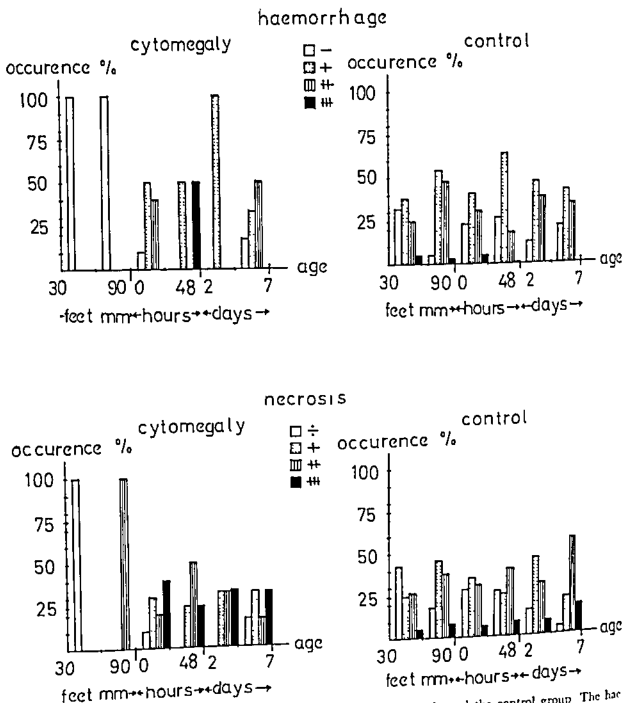


Fig 4 The morphology of the foetal cortex in the cases of cytomegaly and the control group The haemorrhage and the necrosis are quantitated in four degrees and indicated in per cent

tion between adrenal cytomegaly, islet hyperplasia, and Leydig cell hyperplasia in several of her cases of cytomegaly We only found four cases of insular hyperplasia of the pancreas, and two cases of Leydig cell hyperplasia without indication of maternal diabetes or Rh-immunization, but this could imply an association

Some authors suggest that the cytomegalic cells are a manifestation of neoplasia (12, 25, 30), owing to a resemblance to tumours originating from the adrenal cortex (12) In one case, *Sherman et al* (1958) found similar cellular changes in plexus chorioideus the lungs, and the pancreas, and they suggested that it might be metastases of the cytomegalic cells in the adrenals *Oppenheimer* (1970) found cytomegalic cells in

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HISTOLOGY OF THE RAT ACHILLES TENDON BEFORE AND AFTER TENDON RUPTURE

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The Achilles tendon of the rat was studied before, immediately after, and 1-8 days after experimental rupture of the tendon which was induced in wild brown rats The specimens were stained with haematoxylin eosin, alcian blue, and toluidine blue The tendon ruptures proved to be independent of degenerative changes Immediately after the tendon rupture very scanty changes were found, in the form of slight fraying between the fibre bundles and a slight increase of the wavy structure Through the first 24 hours these changes increased At the same time, disintegration of the individual fibres occurred, with slightly reduced stainability and in places pyknotic nuclei It is concluded that these findings represent the mildest changes that may be expected in a biopsy specimen from a previously healthy tendon which has been subjected to a purely traumatic subcutaneous rupture On the basis of the present findings and of previously reported experimental studies it is concluded that the changes described elsewhere following subcutaneous rupture of the human Achilles tendon have not necessarily been present prior to the rupture Consequently, the author cannot accept the biopsy findings from human cases as a convincing link in the argumentation that previous degeneration is a necessary condition for subcutaneous rupture of human tendons

Arguments advanced on the basis of the theory 'a healthy tendon never ruptures' have been founded largely upon results of histological studies of biopsies in cases of subcutaneous tendon rupture (1, 4, 5, 7, 8)

Another argument in favour of this theory has been that tendon rupture could not be induced experimentally (4, 6 10, 14, 15) As experimental tendon rupture did prove possible in a series of experiments performed by the author — not yet published — it seemed reasonable to supplement the experiments by histological studies

The experiments were performed on the Achilles tendon of anaesthetized rats in a material testing machine without previous injury to the tendon The tensile strength experiments were carried out on albino rats as well as upon the closely related wild brown rats Tendon rupture could be induced only in adult brown rats

The problems under consideration in the present study were (1) whether previous degeneration was a necessary condition for the experimental tendon rupture, and (2) whether the histological changes found after experimental tendon rupture can help in the

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interpretation of the histological changes previously described following rupture of the human Achilles tendon

MATERIAL AND METHODS

A total of 157 tendons were studied histologically

A control group was made up of 20 tendons from small albino rats (weighing about 100 g), 20 tendons from large albino rats (about 350 g), and 10 tendons from recently captured large brown rats

Tendon rupture group The author studied 82 Achilles tendons from large rats captured a maximum of two weeks previously and 13 tendons from large brown rats kept in cages for 3 months after being captured

Amputation group Four groups each of 3 tendons, were investigated on the 1st, 2nd, 4th and 8th day after the tendon rupture, a total of 12 tendons To perform the experiment the femoral condyle and the os calcis were subjected to wire traction, and prior to loading the lower leg was cut except for the Achilles tendon To avoid the subcutaneous position of the tendon after tendon rupture and wound closure, the tendon stump was bent upwards so that it was situated between the soleus muscle and the deep muscles of the calf The procedures were carried out under sterile conditions

Immediately after the rupture experiment or, in the amputation group and the control group, immediately after the rats had been killed, the tendons were fixed in formalin The fixation period varied from 24 hours to several weeks After deparaffinization the sections were rinsed in water for 5 min and thereafter stained with haematoxylin eosin, alcian blue, or toluidine blue, and in a number of cases also by the method of van Gieson Hansen

The alcian blue staining (0 5 per cent in 3 per cent acetic acid) was continued for 30 min After rinsing in distilled water for 5 min the sections were stained for 1 min with safranine (0 25 per cent in 0 125 N HCl) and treated with alcohol after immersion in water

The staining time for toluidine blue (0 1 per cent in 30 per cent alcohol) was 8 min After two rinses in distilled water the preparation was treated with alcohol

The above staining times had been found to be optimal

RESULTS

Histology of the Rat Tendon

Proximally the tendon fibres lay parallel, only separated by elongated fibrocytes On staining with alcian blue-safranine this part stained a faint pink On toluidine blue staining slightly metachromatic strands could occasionally be seen in the pericellular areas

Distally in the tendon there was a gradual transition to tissue reminiscent of cartilage (Fig 1) The fibre bundles were still longitudinal, while the slender cells, almost devoid of cytoplasm, became round, with pale cytoplasm surrounding a dark nucleus Closer to the insertion the cells were arranged in columns Distally, the fibres were alcian positive viz blue With toluidine blue this area gave a faintly metachromatic staining

The peritendineum was made up of loose connective tissue with few layers of cells but occasionally there was considerable proliferation without any changes of the tendon tissue proper

In most cases a small part of the muscle was included in the biopsy No abnormalities of the muscle were observed

No changes were found in the vessels within or around the tendon Cyst formation was never found

Degenerative changes or signs indicating degeneration were not found apart from the fact that in about half the preparations — evenly distributed in the various groups — there were foci halfway up the tendon (Figs 2 and 3) These foci resembled most of all cartilaginous metaplasia without any sharp demarcation from the surroundings There were occasional sharply defined alcian negative areas which were at the same time metachromatic These areas stained very dark

Fig 1 (top left) Insertion of the rat Achilles tendon into the calcaneus Posterior to the Achilles tendon the plantaris tendon is seen Alcian blue × 13

Fig 2 (bottom left) Alcian blue as well as *Fig 3* (bottom right) toluidine blue show focal changes in the middle third of the rat Achilles tendon × 40

Fig 4 (top right) Proximal tendon stump 4 days after tendon rupture showing disintegration and waviness of the fibres and pyknotic nuclei van Gieson Hansen × 61

non rupture groups, the changes were of the same nature as those seen at an early stage after rupture of human tendons

The present study did not demonstrate tendon lesions that could explain the experimental tendon ruptures Previous degeneration was not a necessary condition for experimental rupture It must be concluded that other properties in the experimental animals were decisive, properties which presumably have to be related to their conditions of living (wild *versus* domesticated) All previous tensile strength experiments (4, 6, 10, 14) have been performed on domesticated, fairly young animals except for one experimental series on trained rabbits (15) The failure site has never been in the tendon

A short time after the tendon rupture — within 24 hours — there was further fraying of the fibres, a slightly reduced stainability, and mild nuclear pyknosis The present study did not show any definite necrotic changes following tendon rupture Whether the latter should apply also to subcutaneous rupture of normal human tendons is doubtful In the first place, the Achilles tendon in the rat is very thin (0 5 × 1 5 mm) This increases the possibility of survival of the tendon tissue by diffusion In the second place, the tendon, in the present experiment, had to be placed in loose connective tissue in the upper calf instead of remaining *in situ* inside a tendon sheath This sheath is not lined with synovial membrane, it is true, but is nevertheless quite well defined, both in rats and in man In man this tendon sheath is nearly always intact following subcutaneous tendon rupture (2) At operation the tendon is found to be oedematous, and the space between the tendon ends is filled with haemorrhagic fluid or with fibrin The possible necrotic action of this oedema and the attendant venous congestion could not be reproduced in the present experiments

Mere cutting of the tendon cannot be expected to produce any pathological lesions *Long & Vernstein* 1966 (8) have studied the microscopic appearances 1–14 days after cutting rabbit tendons Even at the end of 14 days they found the tendon tissue to be normal except for a few strands of granulation tissue invading from the cut surface

In an experimental series in which rabbit Achilles tendons were crushed with an arterial clamp and the wound closed, *Palla* (11) found that as early as 15–24 hours later the fibrillar structure was almost abolished and that the nuclei were hardly visible In other words, tendon tissue may respond rapidly to severe injury

The fraying and disintegration of the fibres as well as the more wavy structure, the reduced stainability, and the slight nuclear pyknosis found in the present study must, therefore, be considered the mildest changes that may be expected in a healthy tendon which has been exposed to such violence that it has ruptured

Accordingly, it is unlikely that the changes — disintegration of collagen bundles partial absence of nuclei, and almost structureless areas described even during the first 24 hours after subcutaneous rupture of human tendons (1, 4, 5, 7, 8) — have always been present *prior to the* rupture Moreover, more severe changes at this juncture may have been caused by a previous, partial rupture (9)

The specimens were kindly prepared in the *University Institute of Pathological Anatomy, Rigshospitalet, Copenhagen, and in the Bone Laboratory, Orthopaedic Hospital, Copenhagen*

The studies were aided by a grant from *Miss P A Brandt's Foundation*

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with haematoxylin eosin and had presumably contained calcium although actual bone formation was not found. The remaining — peripheral — part of the focus stained like the distal cartilaginous part of the tendon, the alcian positive and metachromatic areas being situated mainly in close relation to the cell membrane.

Differences Between the Groups

The differences between the groups were slight and it was only in the amputation group that they were marked enough to allow distinction from the other groups. In making the comparison emphasis was laid particularly upon reduced stainability of the fibres, the waviness of the fibres, the splitting of the fibres, the appearance of the nuclei, and the stainability of the tissues with toluidine blue and alcian blue.

In the amputation group there was fraying of the fibre bundles, disintegration of the individual fibres into smaller units. The stainability with haematoxylin eosin was somewhat fainter and in places the nuclei were irregular and pyknotic. A decrease in the number of cells was not observed. The stainability with alcian blue and toluidine blue did not alter (Fig. 4).

As a rule there was slight infiltration with round cells and granulocytes, but there was no clinical evidence of infection. The changes were present already at the end of 24 hours and after that grading was impossible on the basis of the duration from the experiment until the removal of the tissue specimens. The preparations gave no reason to believe that further grading within the time limit of 8 days would have been possible even with a considerably larger number of experiments.

Distinction between the groups with tendon rupture and the control groups could be based only upon a somewhat more pronounced fraying and a somewhat more wavy structure of the fibres in the former group. The difference was not so marked that each individual preparation could be classified thereby.

It was impossible to distinguish between the small and the large domesticated rats or between the recently captured brown rats and the brown rats which had been kept in cages for 3 months. Not even the presence of cartilaginous foci could distinguish between the groups.

The above mentioned cartilaginous foci were never found at the site of the rupture, which was invariably 0–2 mm from the insertion (the Achilles tendon in the rats is 10–15 mm in length).

DISCUSSION

The common finding of focal changes in the free part of the tendon was surprising. These areas were cartilaginous, at times with calcification. The explanation is presumably the special anatomy in the rat, viz. the strong plantaris tendon crosses the Achilles tendon to continue behind the calcaneus down into the sole. This produces a pressure force which according to *Ploetz* (12) is able to induce cartilaginous metaplasia. As already stated the rupture did not in any case occur in this area. In other words, the cartilaginous metaplasia has not resulted in mechanical weakness of the tendon in this area.

The histochemical staining methods afforded information concerning the localization of acid mucopolysaccharides, but the studies would have had to be greatly extended in order to ascertain which mucopolysaccharides were present. The presence of mucopolysaccharides in tendon tissue is taken to indicate degeneration (3). In cartilaginous tissue, however, metachromasia is a normal finding (13, 16) and this corresponds to the distribution of metachromasia seen in the rat tendons. The distribution and intensity of staining was the same in the various control groups and in the tendon rupture group.

The changes demonstrable immediately after rupture were slight and consisted in mild splitting and an increased occurrence of wavy fibres. Although the changes do not permit a distinction between the rupture and

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AN AUTORADIOGRAPHIC STUDY OF THE NORMAL LYMPHOID CELL DECAY IN THE MESENTERIC LYMPH NODE OF THE MOUSE

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The cellular decay in mensteric lymph node cell suspensions was studied by combining nigrosin dye exclusion and ^{3}H thymidine autoradiography The labelling pattern of non viable cells was followed during a period of 14 days after injection of a single dose of ^{3}H thymidine into NMRI mice 30 to 45 days of age The decaying cells were divided into DNA synthesizing and non DNA synthesizing cells The decay of DNA syntresizing cells partly reflects a decay of blast cells proliferating *in situ i e* germinal centre cells with a decay rate of 20 per cent The majority of cells decaying in the lymph node seems to be carried to the organ by the blood or lymph stream The results are discussed on the basis of knowledge of lymphoid cell migration streams between the various lymphoid organs and the existence of short lived lymphocyte populations

To-day it is well established that the lympho cyte production in the spleen and lymph nodes is insignificant compared to the inten sive production rate in the thymus and the bone marrow (1 2 7 8) at the same time the rate of lymphoid cell decay in the spleen and lymph nodes is rather high (3, 14), sug gesting that these lymphoid organs selectively remove worn out lymphoid cells from the blood and/or lymph tract

The present work tests this hypothesis and gives information about the kinetics of lym phoid cells decaying in the lymph node using a new method which combines ^{3}H thymidine autoradiography and the nigrosin dye exclu sion technique (5)

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MATERIAL AND METHODS

The experimental animals were 30-40 days old mice of both sexes from a non indbred NMRI strain They were housed in plastic boxes under standard conditions The animals were injected intraperitoneally with 5 micro c/g ^{3}H thymidine (Specific activity 5 0 c/mmol Radiochemical Cen ter, Amersham England) Groups of three mice were killed at intervals ranging from half an hour to 14 days after the injection The mice were sacri ficed by cervical dislocation and the mesenteric lymph node removed Nigrosin dye exclution test (DET) lymphoid cell smears, and autoradiogra phic procedures were performed as previously de scribed (4 5)

Cell Typing

Lymphoid cells from smears not exposed to DET were examined and divided into groups on the basis of the mean cell diameter cells with a dia meter of 7 microns or less and cells with a dia meter above 7 microns The very small number

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jection is one the same level in the two cell fractions Not even the most heavily labelled nigrosin stained cell fraction shows a selective rise indicating that no ^{3}H thymidine irradiation effect exists during the first 48 experimental hours A few non viable cells (0 1 to 0 2 per cent not included in Fig 1) had more than 50 grains per cell Such heavy labelling was never found in the viable cell group and the decay might be due to irradiation

Figure 2 shows the labelling index of all lymphoid cells (TLI) and the labelling index of the nigrosin stained lymphoid cells (SLI) as a function of time after injection of ^{3}H thymidine The curves marked A and B are drawn through the mean labelling index of three animals at various time intervals after H thymidine injection

Initially TLI (curve A) is about 1 5 per cent From this value the percentage of labelled cells increases during the first 48 hours to reach a level of 10 per cent during the rest of the experimental period Half an hour after the injection SLI (curve B) is 3 per cent and increases through the first 48 hours to a peak of 23 per cent Then a sharp decline to 12 per cent follows on the 3rd and 4th day This is followed by a new increase to 22 per cent on the 5th day after which the labelling index falls to 14 per cent 14 days after injection of ^{3}H thymidine Half an hour after injection SLI (3 per cent) reflects the decay of cells in the DNA synthesizing cell population prolifering locally in the lymph node i e germinal centre activity The fraction of decaying lymphoid cells in the lymph node is 10 per cent Thus 1/33 × 10 per cent = 0 3 per cent of the total lymphoid cell decay in the lymph node belongs to the DNA synthesizing cell population As DNA synthesizing lymph node cells amount to at least 1 5 per cent (the initial value of TLI), about $\frac{0.3 \times 100}{1.5}$ = 20 per cent of this cell fraction decays locally in the mesenteric lymph node Later in the experimental period the majority of labelled decaying cells are derived mainly from non DNA synthesizing and DNA synthesizing cells with origin both in the lymph node and in other haemopoietic tissues (see discussion)

Figure 3 shows the ratio between SLI and TLI calculated from individual animals and expressed in per cent At all time intervals examined this ratio is above 100 per cent During the experimental period the cell populations in the mesentric lymph node are in

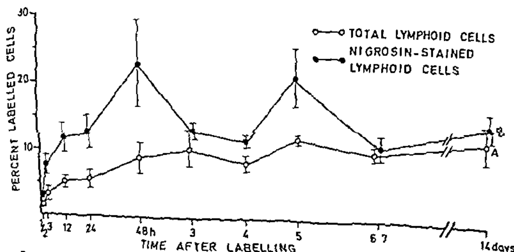


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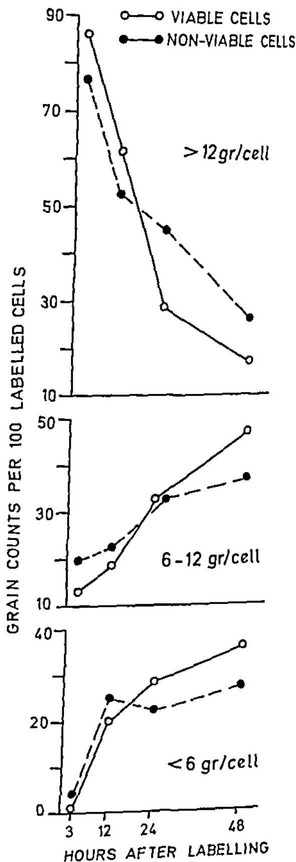


Fig 1 The distribution of viable and non-viable cells containing varying degrees of labelling The curves are drawn through the mean grain counts from two animals sacrificed at various time intervals after the injection of ^{3}H thymidine

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of reticular cells found in these preparations were not typed

Autoradiographical Analysis

Cells with less than three grains above the background area were considered unlabelled The labelling index of all lymphoid cells – viable and non viable – was estimated by counting 2000 labelled cells from each lymph node smear The labelling index of the non viable (*nigrosin stained*) cell fraction was determined by counting 1000 non-viable cells per lymph node smear The percentage of non-viable cells was calculated from countings of 2000 to 3000 cells, *i e* at least 200 nigrosin stained cells were registered in smears from a single lymph node To estimate a possible irradiation induced cell damage, comparative studies of mesenteric lymph node suspensions from mice not injected with the isotope were performed Furthermore, grain counts of 100 viable and non viable cells were made in order to establish if there were a selective decay in the heavily labelled cell fraction

RESULTS

Differential counts performed on smears not exposed to dye exclution test (DET) showed that about 95 per cent of the cells belong to the small lymphocyte catagory with a cell diameter of 7 microns or less About 5 per cent of the cells had a diameter above 7 microns, and less than half a per cent were reticular cells

The percentage of nigrosin stained lymphoid cells in the smears exposed to DET was found to be 10 per cent ± 1 8 per cent*), *i e* at the same level as in mesenteric lymph node suspensions from mice not injected with ^{3}H-thymidine (3)

Figure 1 shows the distribution of viable and non-viable cells with varying labelling intensity Each point of the curves represents the mean grain count from two animals The intensity at various time intervals after in-

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*) s d

decay in the thymic blast cell population was found in a recent study (5) Calculations on the decay of DNA synthesizing germinal centre cells indicate that about 20 per cent of these cells probably decay in the last part of the S phase or in the G phase of their generation cycle Using the same technique on thymus cell smears we found that about 8 per cent of the thymic blast cell population decays *in situ* (5)

It is well known that cellular migration streams exist between the bone marrow and the thymus on the one hand and the spleen and lymph nodes on the other (for review see 10) The present work indicates that at least some of these migrating cells are in fact non viable and decay in the lymph nodes This point of view has been stressed years ago (*Heiberg* 1923) From studies by *Everett et al* (1964 and 1967) it is known that 100 per cent of the bone marrow lymphocytes 95 per cent of the small thymic lymphocytes 35 per cent of the blood lymphocytes and about 25 per cent of the mesenteric lymph node lymphocytes are short lived cells with life spans of 3 to 5 days The fate of these short lived lymphocytes is partly unknown at the present time

The majority of decaying cells probably are of the short lived type The greater part of lymphocytes decaying at the first peak (day 2) may well be bone marrow derived cells which are even more short lived than the short lived thymic cells (8) indicating that the decay at day 5 may represent short lived thymus derived cells

This hypothesis fits well into recent studies which have shown that bone marrow and thymus derived lymphoid cells interact in the peripheral lymphoid organs during immune reactions (6 13) If the cells meet the antigen to which they are genetically primed they will proliferate and differentiate to plasma cells and long lived re circulating memory cells If on the other hand, the short lived lymphocytes do not meet the appropriate antigen – and this of course will be the most common situation – they may decay in the lymphatic tissue

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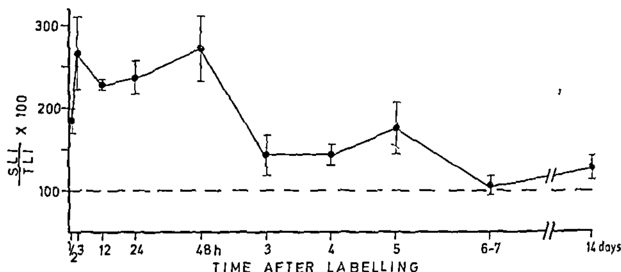


Fig 3 The ratio $\frac{\text{non viable cell labelling index (SLI)}}{\text{total lymphoid cell labelling index (TLI)}} \times 100$ calculated from individual animals as a function of time after injection of ³H thymidine Bars represent ± SEM calculated from groups of three mice

a steady state with a constant rate of cell proliferation a constant flow of cells entering and leaving the lymph node the average per cent of decaying cells being about 10 Therefore any difference between SLI and TLI reflects selective movements of non viable and/or viable cells entering or leaving the lymph node Figure 2 and 3 shows that SLI is above TLI at all time intervals The regular shape of curve A makes it probable that the lymph node – expressed in per cent – receives more non viable cells than viable cells The decay of these blood or lymph borne cells is especially pronounced day 2 and day 5 after injection of ³H thymidine

DISCUSSION

The percentage of nigrosin stained lymphoid cells in the lymph node smears corresponds to the number of nigrosin stained lymphoid cells from mesenteric lymph node cell suspensions studied in a previous work (3) No ³H thymidine induced cell damage could be demonstrated by comparing grain numbers in the viable and the non viable cell fractions (Fig 1) However in a small fraction of nigrosin stained cells (0 1 – 0 2 per cent) more than 50 grains per cell were found – a labelling intensity never seen in viable cells – indicating some irradiation induced damage among heavily labelled cells The size distribution of lymphoid cells equals that seen by *Matsuyama et al* (1966) in mesenteric lymph node smears from C Bl and AKR mice These authors examined the labelling of various lymphoid tissues after a dose of ³H thymidine comparable to the dose used in the present work They exposed their autoradiographs up to 120 days Therefore they found rather high labelling indices (about 30 per cent) They concluded – as done here – that only a minor degree of radiation damage occurred which might be attributed to the rather high dose of ³H thymidine used

Three per cent of the decaying cells were labelled within half an hour after the injection indicating a partial decay among DNA synthesizing germinal centre cells (Fig 2) This finding is in agreement with the work of *Fliedner et al* (1964) who found labelled nuclear fragments (tingible bodies) within the cytoplasm of germinal centre macrophages of the rat spleen 30 minutes after injection of ³H thymidine The authors conclude that tingible bodies are formed mainly from dying germinoblasts A similar early

decay in the thymic blast cell population was found in a recent study (5) Calculations on the decay of DNA synthesizing germinal centre cells indicate that about 20 per cent of these cells probably decay in the last part of the S-phase or in the G phase of their generation cycle Using the same technique on thymus cell smears we found that about 8 per cent of the thymic blast cell population decays *in situ* (5)

It is well known that cellular migration streams exist between the bone marrow and the thymus on the one hand and the spleen and lymph nodes on the other (for review see 10) The present work indicates that at least some of these migrating cells are in fact non viable and decay in the lymph nodes This point of view has been stressed years ago (*Heiberg* 1923) From studies by *Everett et al* (1964 and 1967) it is known that 100 per cent of the bone marrow lymphocytes 95 per cent of the small thymic lymphocytes 35 per cent of the blood lymphocytes and about 25 per cent of the mesenteric lymph node lymphocytes are short lived cells with life spans of 3 to 5 days The fate of these short lived lymphocytes is partly unknown at the present time

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THREE-DIMENSIONAL RECONSTRUCTION IN HISTOLOGY

A Short Survey of Available Methods and Description of a New Technique

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A new technique for three dimensional reconstruction from serial sections is described. The method implies transfer of drawings of histological sections to acryl plates and after gluing the plates together, a highly transparent block is produced, in which details can easily be examined. This technique can be characterized as quick, handy and non expensive. As a choice between the available methods is necessary in order to meet the special demands of the investigation in question, a short survey of the present methods is given.

In histology it must be essential to know to which extent we are allowed to interpret our two-dimensional histological slide into the four-dimensional world of spacial structures changes in relation to time. In pathology the introduction of repeated biopsies—often in the form of needle biopsies from parenchymatous organs—has been of paramount importance for our understanding of the dynamics of many pathological conditions. But is our conception of the spacial relations in the tissues on this base correct and thereby relevant for our understanding of pathogenesis? Although hepatic histology has been studied since the earliest days of microscopy, it was not until 1948 (5) (6) (7) that the conception of livercell cords was abandoned and the liver cell plates accepted as the structural unit of the parenchyme.

We do not have any technique with which we can study lumps of tissues as such at a magnification as given by the microscope. Not too dense tissue, *e. g.* lung tissue has with success been studied *in sections* 200-300 µm in thickness after simple staining and mounting in Permount (29). Focusing up and down in the block, gives an impression of the spacial configuration, but does not allow this impression to be fixed for further investigation and communication. Better results are obtained if the specimen is embedded in a mixture of Kolphonium and Alcarin with a resulting refraction index similar to that of the main constituent of the tissue (2). However the content of stained elements will limit the thickness of the block if sufficient light has to come through the *specimen*. A special group of reconstructions are based on the filling of hollow structures—most often vessels—with a material and subsequent corrosion of the surrounding tissue. Several substances have been used for injection, one of the more suited being vinyl acetate. KOH 20 per cent is an excellent digestive for most soft tissues (16) (28). The major problem when applied to tubular structures like vessels or bile ducts is the filling

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of the smallest radicles Often the necessary injection pressure causes extravasation The lower limit of lumen to be filled in this way seems to most investigators to be about 100 µm (3)

For most investigations we have to work with ordinary histological sections of usual thickness The compensation for these thin sections must be that we base our opinion and description on a multitude of them, best in the *form of serial sections* But if the anatomical structures to be investigated are of greater complexity, the human visual system is incapable of retaining such complex topographical data and we have to make some sort of reconstruction The type of reconstruction to be used depends on the character of the investigation in question Sometimes a three-dimensional reconstruction is necessary A new technique for this is presented

MATERIALS AND METHODS

A New Technique

During the search for a more suitable transparent material than the below mentioned for three-dimensional reconstruction a great number of plastic products were examined The demands to the material were the following a) It should be as transparent as possible b) It should be easy to glue without loss of transparency c) It should be commercially available in plates of a thickness fit for this purpose d) It should be cheap e) It should be easy to handle *i e* not demanding complicated machinery high pressures or heat for the gluing process and be easy to polish The material fulfilling these demands to the highest degree was found to be acryl

The reconstructions made by the author have been of portal tracts in liver tissue (14) For these investigations a total magnification of 150 200 times microscope + drawing device) is very suitable As the acryl is available in plates of 1 mm thickness this matches with a thickness of the serial sections of 5 µm

A complete series of sections comprising 200 300 cuttings of the selected portal space is produced The structures of interest are drawn on tracing paper from the slides by the aid of a Leitz drawing device Points of reference are found by measurement of distances from the section borders These drawings are then transferred to acryl plates

Now two different ways of handling the plates have been used The first implies punching of the tubular structures (vessels and bile ducts) which after gluing the plates together are filled with dyes of different colour (Fig 1) The second implies simple colouring of the drawings with a water colour pencil before gluing the plates together Following this technique nothing can be seen, when the resulting block is viewed directly from the side It has to be tilted a little (Fig 2) This disadvantage is not found following the first described technique but the price for this gain is—in the author s opinion—much too high in form of the very laborious and time consuming work it is to punch out the tubular structures

Acryl is soluble in di-chlor ethan and this solvens or a cement produced by solving small pieces of acryl in di-chlor-ethan can be used to glue the plates together The advantage is cheapness the disadvantage is that di-chlor-ethan is very volatile, so one has to work fast Mainly for this reason the author has changed to another glue I C I Tensol No 7 ® This is a two component glue, one component being a polymerizer of the other This method is more expensive than the di-chlor-ethan method, but more easy to work with and giving excellent results

Only slight pressure on the block is necessary after the gluing The following day the block is hard and can be polished

DISCUSSION

The most important methods of reconstruction from serial sections may be listed under the headings

A Graphic
B Photographic
C Three Dimensional

A Graphic

A very simple method is the following, as stated by Wendell Krieg "One merely projects on a sheet of paper the outlines of any structure in a series of sections and draws its shifting trace in proper relation, so that one obtains a contour map of the object It is easy to transform a contour map into a shaded topographical map by visualizing the light as coming from the upper left and shading" This method has been used in the field of neuroanatomy with brilliant results by Wendell Krieg himself (15) and also by others It demands some practice, but may fulfil the demands of many types of investigation (20)(27) A more satisfying method

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THREE-DIMENSIONAL RECONSTRUCTION IN HISTOLOGY

A Short Survey of Available Methods and Description of a New Technique

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A new technique for three dimensional reconstruction from serial sections is described The method implies transfer of drawings of histological sections to acryl plates and after gluing the plates together, a highly transparent block is produced, in which details can easily be examined This technique can be characterized as quick, handy and non expensive As a choice between the available methods is necessary in order to meet the special demands of the investigation in question, a short survey of the present methods is given

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We do not have any technique with which we can study lumps of tissues as such at a magnification as given by the microscope Not too dense tissue, *e g* lung tissue, has with success been studied in sections 200 300 μm in thickness after simple staining and mounting in Permount (29) Focusing up and down in the block, gives an impression of the spacial configuration, but does not allow this impression to be fixed for further investigation and communication Better results are obtained if the specimen is embedded in a mixture of Kolphonium and Alcarin with a resulting refraction index similar to that of the main constituent of the tissue (2) However, the content of stained elements will limit the thickness of the block if sufficient light has to come through the specimen A special group of reconstructions are based on the filling of hollow structures—most often vessels—with a material and subsequent corrosion of the surrounding tissue Several substances have been used for injection, one of the more suited being vinyl acetate KOH 20 per cent is an excellent digestive for most soft tissues (16)(28) The major problem when applied to tubular structures like vessels or bile ducts is the filling

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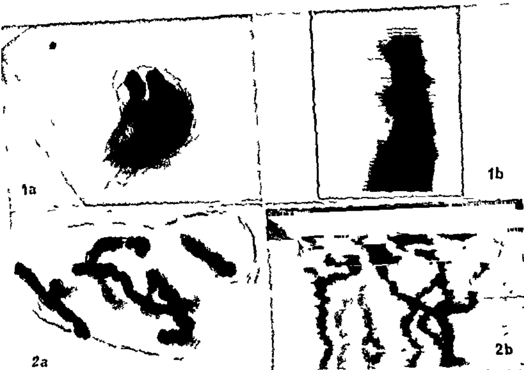


Fig 1A The block is seen from the top The borders of the portal space (the limiting plates) are indicated by a single line The portal artery (red) and bile duct (green) are seen in front of the portal vein branch (blue)

Fig 1B The block is viewed directly from the side

Fig 2A The block is seen from the top There is a little branch of the portal tract to the right as indicated by the limiting plate Artery red, vein blue and bile ducts green

Fig 2B The block is seen from the side in a little tilted position The bile duct in the portal tract branch (upper right part of the picture) is without connection to the bile duct of the main tract

method also apply to this material Stacking the glass plates a partially transparent model is produced On account of the refraction of light from glass plate to glass plate the transparency is not quite satisfying but if the whole stack is placed in a glass box containing glycerol then the model is easily trans illuminated

The glass plate method is quick and cheap and it is possible to perform measurements on the model The disadvantages are Looking directly from the side one cannot see anything It is necessary to tilt the block a little and so examining it under an oblique angle The suspension in glycerol is a little unhandy

When the glass plate method is compared with the author's method many similarities are evident But the new technique has some advantages to the old one, the most important being a much higher degree of transparency, and no disadvantages except a little higher cost in materials The method is not much more time consuming and the resulting block is handy Details can easily be examined and measurements performed

This work was supported by a grant from Købmand i Odense Johann og Hanne Weimann f Seedorffs legat

is the perspective graphic reconstruction which implies perspective distorsion of the separate drawings from a rectilinear grill to a perspectively distorted grill (17) (10)

The graphic methods are among the less time consuming i e less expensive The main disadvantages are a) You are bound to the angle of view chosen at the beginning of the reconstruction b) Measurements on the reconstruction are difficult or impossible

B *Photographic*

Microphotographic transparencies superimposed with spacings proportional to the corresponding planes of the object form a stereosolid or photostereosynthesis In this way using Translite photographic paper, *Hegre* (11) made a reconstruction of a pig embryo The technique has been perfected using polaroid transparency emulsions and three layered subtractive colour emulsions allowing recording of three different planes of the object on the same photograph by the means of coloured filters (4) One of the major problems in reconstruction from serial sections is the following Identification of points of reference It was however in a very elegant way solved by *Hegre et al* (12) (13) for many types of photographic reconstructions They photographed the tissue block in fixed position after discharging the cut section and staining the exposed surface of the block An automated device for this technique has been described by *Postlethwait et al* (24) Unfortunately the method is based on a very quick surface staining technique which has not till now included the general histological stains It has been used for reconstructions of embryoes lung tissue and spinal cord in the form of cinematography in which time is substituted for height giving an impression of proceeding through the tissue block Some investigators (21) have found the combination of three dimensional reconstruction and cinematography useful The latest development in the photographic way of reconstruction seems to be an electro optical device invented by *Roger Lannes De Montebello* (18) in which the input is photographs on a 16 mm film strip and the output is a three dimensional virtual synthesis which stands in space, visible within 360° azimuth and 80° elevation

In general photographic reconstructions are rather expensive in equipment and materials

C *Three Dimensional*

In this group the old wax plate method is classic (26) If we trace the successive outlines of a structure as they project from a series of sections on a wax plate, made as many times thicker than the section as the number of units of magnification of the image we have when we stack the plates a magnified simulacrum of the object after the stepped outlines are smoothed off by local heating or sculpturing Minor modifications of this method have been proposed (25) (19) When *Johannes Fibiger* in his earliest works with the spiroptera tumour of the rat tried to identify the worm it was by reconstruction in cardboard from serial sections (9) A quite similar technique was used by *Takahashi & Hyama* (27) in preparing a cardboard model of a portal space

The methods based on wax or cardboard are quick and non expensive The disadvantages are The model being solid and opaque does not show what is inside any shape Outside structures mask deeper ones Weak or detached parts might be held together with wire

Several transparent materials have been used *Pedler & Tilly* have used sheets of expanded polystyrene in reconstruction of plasmamembrane profiles from electron microscopic photos The transfer of outlines from the photos to the polystyrene was done by a specially designed pantograph (22) Also celluloid has been used but with disappointing results (1) The glass slide method of *Hans Popper* has given excellent results (23) (8) (21) The principle is drawings on glass plates from histological sections and finally piling of the plates The considerations regarding magnification and thickness of the plates mentioned under the wax plate

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A CASE OF ABNORMAL INTRAHEPATIC BILE DUCT ARRANGEMENT SUBMITTED TO THREE DIMENSIONAL RECONSTRUCTION

M JORGENSEN

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A case of abnormal intrahepatic bile duct arrangement, which in all probability should be classified as congenital hepatic fibrosis, was submitted to three-dimensional reconstruction This proved that the duct system of the portal tracts had the form of curving plates This unusual architecture is compared with the embryological development of the intrahepatic bile duct system On this base a hypothesis of the pathogenesis of the abnormal findings is presented

CASE REPORT

A 24 years old healthy woman was admitted to hospital for delivery on acount of her preceding obstetric history She had had two stillborn babies with meningocele and club-foots and one hydrocephalic baby dying 3 min after birth Autopsy was not performed in these three cases The actual pregnancy was uneventful and the birth took place at term [illegible]

[illegible] The two main diagnosis were large hydrocephalus and polycystic disease of the kidneys In addition abnormal development of several endocrine organs was recorded The liver was grossly normal, weight 133 g The extrahepatic bile ducts were normal. The liver histology showed unusual traits The liver parenchyme seemed normal as to the architecture and cytology No bile stasis was present. Apart from islands of haematopoietic cells normal for the age no infiltrates were seen The central veins appeared normal The portal spaces were moderately enlarged with an increased content of mature collagen No inflammatory exudate was found The vessels in the portal spaces were normal, especially were the portal vein branches in no area hypoplastic or missing The most astonishing feature was the bile ducts, which to a very large extent were cut longitudinaly (Fig 1) and often displayed a circular or semicircular course around veins (Fig 2), arteries (Fig 3) or a core of dense collagen tissue (Fig 3) In many places the duct-like structures only consisted of one row of epithelial cells, many of which showed overt degenerative changes In other places two rows of ductal cells were seen round a lumen (Fig 4) The longitudinaly cut ducts had normal connections to the [illegible] these portal spaces and no cysts were seen The picture of marginal bile duct proliferation as in cases of inhibition of the bile flow was never observed

According to stereometric principles these "longitudinaly cut bile ducts" should in reality represent cut plates To prove this, a three-dimensional reconstruction of a portal space was made (the major one in Fig 3) The technique used has previously been described (3) It implies transfer of drawings from serial sections to acryl plates and gluing of the plates to produce a transparent block The te-

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Fig 5 Reconstruction of the portal space shown in fig 3 The ducts are indicated by hatching in black The duct system has the form of a cylinder round the hepatic artery branch (red) The intact front wall of the cylinder is shown To the right a normal tubular duct is leaving the cylinder To the left the limiting plate is seen with the origin of a tubular duct The veins are indicated in blue

Fig 6 A defect in the backwall of the cylinder and disappearance or break up of the frontwall

sult was that to be expected The ductular system did have the form of curving plates In Fig 5 it has the form of a complete cylinder round the hepatic artery branch By following the cylinder down through the block it can be seen that it gradually breaks up (Fig 6) and finally ends in a few ordinary looking tubular ducts

DISCUSSION

What is the interpretation of this 'ductal plate System'? And is it really plates and not closely packed tubular ducts? To answer the last question one needs optimally preserved tissue, and not as here autopsy material, and semi thin sections of a thickness less than 1 μm Thus the question can not be answered via this investigation The indications of a congenital malformation are strong The family history, the findings of multiple and severe malformations of other organ systems and the complete abscence of signs of disease in the form of reactivity in the liver tissue of this newborn girl If the classification as a congenital malformation is accepted, no further understanding an be expected except in the light of our knowledge of liver embryology Unfortunately this knowledge, including the development of the intrahe-

Fig 1 Portal and perilobular fibrosis Longitudinally cut bile ducts with connections to the limiting plates In the parenchyme small foci of haematopoietic cells (Van Gieson stain, × 84)

Fig 2 Portal space with bile ducts circling around portal vein branch (Van Gieson stain × 140)

Fig 3 The two small portal spaces in the upper part of the picture illustrates bile ducts circling around a core of dense collagen tissue In the bigger portal space to the right the duct is completely encircling an an artery This space was chosen for three dimensional reconstruction (Van Gieson stain × 84)

Fig 4 Higher magnification of a part of the bigger portal space in fig 3 Cytological details of the duct epithelium are visible (Van Gieson stain × 350)



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BRIEF REPORT

ENDOCRINE CELLS IN THE BRONCHIAL MUCOSA OF HUMAN FOETUSES

Esther Hage

There exist in the body a widespread family of cells in both endocrine and nonendocrine tissues sharing a number of cytochemical and ultramicroscopic characteristics These cells have been given the name APUD-cells derived from the initial letters of their three cytochemical characteristics amine content amine precursor uptake and amine acid decarboxylase (*Pearse* 1969) A common function of the cells is secretion of low molecular weight polypeptide hormone The hormone is believed to be stored in secretory granules, but its synthesis in the cells has not yet been proven in details

The lung endocrine cells are thought to be members of this group of cells and the present study is concerned with the cytochemical characteristics of bronchial endocrine cells especially their amine content and their uptake of amine precursor

Material and Methods

The material comprises lungs from a total of 35 human foetuses of both sexes removed from the uterine cavity as a part of legal abortion The crown rump lengths of the foetuses of the material range from 53 190 mm (measurements being made on unfixed foetuses which were held in the supine position) Pulmonary tissue was obtained within 5 minutes after abortion and fixed quickly in ice cold 10 [illegible] ouin's fluid for 24 hours

The following methods were applied to paraffine sections

1 HCl basic dyes method for endocrine cells according to *Solcia et al* (1968)

2 Lead haematoxylin (Pb-H as a stain for endocrine cells according to *Solcia et al* (1969 b)

3 For detection of argentaffine cells Diazonium reaction with Fast Garnet GBC or Fast Black K and Zanthydrol reaction (*Solcia et al* 1969 a) Masson Hamperl s argentaffin reaction (*Vialli* 1966, *Singh* 1964)

4 For detection of argyrophil cells Davenport argyrophil method (*Hellman et al* 1960, *Vasallo et al* 1969) Bielschowsky argyrophil method as modified by *Sieter et al* (1965) Bodian's silver method (*Singh* 1962, *Grimelius* 1964) Grimelius silver nitrate stain (1968)

5 In selected cases small pieces of pulmonary tissue were incubated in continuously oxygenated Tyrode s solution containing 25–100 my g/ml of L-dihydroxy phenylalanine (L-DOPA), according to the procedure recommended by *Håkanson et al* (1969) Control pieces were incubated in Tyrode s solution alone

Result and comments

Endocrine cells were only identified in the bronchial mucosa They were situated basally in the epithelium close to the basement membrane The cells were pyramidal or bottle shaped, sometimes forming a shallow cone which might reach as far as to the lumen of the bronchus The cells might be located in the main bronchi as well as in the intrapulmonary bronchi In the smaller bronchi they were usually occurring singly, but sometimes small groups of three to five cells were found at points of divisions of bronchi With the HCl basic d[illegible] c[illegible] P[illegible] a[illegible] ir[illegible] using the argyrophil silver technique the cells were stained by Grimelius silver nitrate stain but neither with the technique of Bodian the technique of Davenport, nor the technique of Bielschowsky After incubation with L-DOPA, several cells exhibited formaldehyde induced fluorescence (dopamine) indicating uptake and decarboxylation of L-DOPA The number of these cells seemed to exceed the number of cells that were positive with the other staining reactions applied Few cells were seen with a small green fluorescence rim near the basis of the cell in the

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patic biliary system is inadequate Although a matter of dispute, the most accepted conception of human liver embryology today seems to be that of *Hans Elias* (1) Regarding the intrahepatic duct system it may be summarized as follows In the first 7 weeks of life no intrahepatic duct system exists In an 18 mm embryo the process starts as a cavitation or tubulation of the muralium duplex or multiplex, i e cell plates of a tickness of two or several cells This transformation from parenchyme to ductal plate and ductal epithelium seems induced or elicited by contact with mesenchyme along the branches of the portal vein and the process starts in the porta hepatis When a duct has reached greater maturity, connective tissue penetrates between it and the parenchyme and "dissects' it out No information seems to be available concerning the fate of the small ducts in the ductal plate which does not reach full maturity They seem to disappear Nothing is known about the factors governing this process

In a very early phase of the development of the intrahepatic bile duct system this system has the form of a plate the ductal plate Some sort of regression arrest — a mechanism accepted in other forms of congenital malformations — may be the explanation of the unusual architecture in this case for unknown reasons a delayed breakdown and disappearance of superfluous ducts

Regarding the relation between this case and established entities of congenital malformations of the liver attention should be drawn to the concomitant polycystic disease of the kidneys Two disorders have a well established connection to this renal malformation It is polycystic disease of the liver and congenital hepatic fibrosis by most investigators regarded as two expressions of the same basic pathological process

Should this case be classified under one of these headings? The lack of cysts exclude the first Regarding congenital hepatic fibrosis many investigators have emphasized the importance of hypoplasia or abscense of portal vein radicles (4) (5) (8), a few deny this concomitant anomaly (2) and some does not mention the vessels at all (6) *Parker* (7) finds hypoplasia of the vessels in one of six cases In this case the vessels were found to be normal The other generally accepted criteria for the diagnosis (4) are fulfilled The characteristic features of the bile ducts i e the longitudinally cutting and circling round vessels are described by several authors (6) or evident from their illustrations in papers on hepatic fibrosis but has not by these authors been further investigated

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BRIEF REPORT

ENDOCRINE CELLS IN THE BRONCHIAL MUCOSA OF HUMAN FOETUSES

Esther Hage

There exist in the body a widespread family of cells in both endocrine and nonendocrine tissues sharing a number of cytochemical and ultramicroscopic characteristics These cells have been given the name APUD cells, derived from the initial letters of their three cytochemical characteristics amine content, amine precursor uptake and amine acid decarboxylase (*Pearse* 1969) A common function of the cells is secretion of low molecular weight polypeptide hormone The hormone is believed to be stored in *secretory granules but its* synthesis in the cells has not yet been *proven in* details

The lung endocrine cells are thought to be members of this group of cells and the present study is concerned with the cytochemical characteristics of bronchial endocrine cells especially *their amine* content and their uptake of amine precursor

Material and Methods

The material comprises lunes from a total of 35 *human* foetuses of both sexes removed from the uterine cavity as a part of legal *abortion* The crown

range

made

supin as obtained within 5 minutes after abortion *and fixed* quickly in ice cold 10 per cent neutral *formaldehyde* 6 per cent glutaraldehyde in phosphate *buffer at pH* 7 4 glutaraldehyde picric acid (*Solcia et al* 1968) and Bouin s fluid for 24 hours.

The following *methods* were applied to paraffine sections

1 HCl-basic dyes method for endocr

of East Black h

and Zanthydrol reaction (*Solcia et al* 1969 a) Masson Hamperl s *argentaffin reaction* (*Vialli* 1966, *Singh* 1964)

4 For detection of argyrophil cells Davenport argyrophil method (*Hellman et al* 1960, *Vasallo et al* 1969) Bielschowsky argyrophil method as *modified by Sievers et al* (*1965*) Bodian s silver method (*Singh* 1962, *Grimelius* 1964) Grimelius *silver nitrate stain* (1968)

5 In selected cases, small pieces of pulmonary tissue were incubated in continuously oxygenated *Tyrode s* solution containing 25–100 my g/ml of *L-dihydroxy* phenylalanine (L DOPA), according *to the* procedure recommended by *Håkanson et al* (1969) Control pieces were incubated in Tyrode s solution alone

Result and comments

Endocrine cells were only identified in the bronchial mucosa They were situated basally in the epithelium close to the basement membrane *The cells* were pyramidal or bottle shaped, *sometimes* forming a shallow cone which might reach as far *as to* the lumen of the bronchus The cells might be located in the main bronchi as well as in the intrapulmonary bronchi In the smaller bronchi they were usually occurring *singly*, but sometimes small groups of three *to five cells were* found at points of divisions of bronchi With the HCl basic dyes method the *cells* gave a purple to violet metachromasia and they were stained dark blue by the Pb-H *None of the* chemical reactions described as positive *for argentaffin cells* could be *observed* in these cells Using the argyrophil silver technique the *cells* were stained by Grimelius silver *nitrate* stain but neither with the technique of Bodian the technique *of* Davenport nor the technique of Bielschowsky After incubation *with L-DOPA* several cells exhibited *formaldehyde* induced fluorescence (dopamine) indicating uptake and decarboxylation of L-DOPA The number of these cells seemed to exceed the number of cells that were positive with the *other* staining reactions applied Few cells were *seen* with a small green fluorescence rim near the basis of the cell in the

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control, not L DOPA incubated specimens The number and distribution of these cells are similar to the number and distribution of cells that were positive with the other staining reactions applied

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TRANSACTIONS OF THE SWEDISH PATHOLOGICAL SOCIETY

Meeting Stockholm November 27 28, 1970

J Hogberg & S V Jakobsson THE EFFECT OF LIPID PEROXIDATION ON LIVER MICROSOMES

The endogenous peroxidation of lipids is thought to be of importance during cell damage in different pathological conditions – Therefore, in order to obtain further information on the enzyme membrane relationship in the liver microsomes, glucose 6 phosphatase (G6Pase), NADH and NADPH cytochrome *c* reductase activities were measured during increasing degrees of lipid peroxidation

NADH and NADPH-cyt *c* red activities showed an initial increase of their activities (when about 5 per cent of the available lipid was peroxidized), but were then more or less unaffected up to the maximal level of peroxidation used (40 *per cent*) *On the other hand the* G6Pase activity after a small initial increase, exhibited a bifasic *curve* including a minimum and a maximum (at about 10 per cent respectively 20 per cent of maximal peroxidation) At higher degrees of peroxidation the G6Pase activity continuously decreased

Kinetic analysis of the apparent V_{max} and K_m for G6Pase showed changes parallel to the activity *curve* The conclusion was drawn that neither addition nor withdrawal of an enzyme inhibitor could solitarily explain the recorded changes in G6Pase *activity After* lower degrees of peroxidation, the addition of NADOC (0 02 per cent.) also restored the basal G6P*ase activity* – Our results strongly suggest that lipid peroxidation primarily affects the membrane and *not* the enzyme

L Erik J Ericsson, J Pontén and B Westermark IN VITRO DIFFERENTIATION AND SPECIALIZATION OF CELL SURFACES IN HUMAN GLIA LIKE CELLS

Human astrocyte like cells were studied ultrastructurally

The *upper surface* of the plasma membrane was smooth, and only occasionally formed coated pits The cytoplasm below this membrane contained densely packed microfilaments Along the *lower surface*, the plasma membrane appeared to be anchored to the microprecipitate through formation of attachment devices Between these attachment devices there was a slit formed or wider space Since thorium dioxide particles added to the medium became concentrated in this space, it could not represent an artifact Small endocytic vacuoles were abundant near the lower plasma membrane which formed many coated pits It appears that the cell's main uptake of macromolecules occurs via the lower surface Microfilaments were rare along the lower surface outside the attachement devices Sometimes portions of cytoplasm from adjacent cells were layered one over another, and adjoining plasma membranes might exhibit junctional devices Hence, the contact inhibited cells were not arranged as a monolayer in all places

These observations indicate that, in a population of glia like cells showing contact inhibition *in vitro* cell surfaces with different fine structure (and presumably different properties and function) are developed

Jan L E Ericsson QUANTITATION OF AUTOPHAGY IN HEPATIC PARENCHYMAL CELLS

In order to create a base line for studies of the autophagosome inducing potential of drugs and toxins on rat hepatocytes, quantitative morphometric and stereological principles were applied for the assessment of volumetric density, collective volume, and absolute number per normal liver cell of secondary lysosomes and autophagic cytosegresomes The results indicated *that* the number of autophagic cytosegresomes (*i e* autophagosomes and „early" autolysosomes) in peripherally *located* hepatocytes was of the order of 5 per cell Calculations of the normal rate of mitochondrial digestion in autophagic vacuoles *suggested* that the whole po-

pulation of mitochondria was replaced in approximately 20 days

By labelling secondary lysosomes in hepatocytes with iron granules and subsequently inducing autophagy through the administration of glucagon (100 μg/100 g b w to rats), the quantitative relationship between primary and secondary lysosomes and autophagic cytosegresomes were established using the morphometric principles mentioned above The results showed that the majority of the autophagic cytosegresomes resulting from glucagon administration developed into ambilysosomes, i e were created through the fusion between secondary (iron containing) heterolysosomes and autophagosomes The findings supported the notion that fusion between preexisting lysosomes and autophagosomes is the mechanism whereby lysosomal enzymes are brought in contact with the sequestrated portions of cytoplasm in the autophagosomes

L Enerback FLUORESCENCE MICROSCOPICAL AND CYTOFLUOROMETRIC DEMONSTRATION OF DOPAMINE UPTAKE BY RAT MAST CELLS IN VIVO

Rat mast cells which normally contain 5 hydroxytryptamine (5 HT) concentrate a catecholamine after the administration of L DOPA This catecholamine uptake was studied by fluorescence microscopy and quantitative cytofluorometry utilizing the formaldehyde condensation method In peritoneal mast cells the subcutaneous injection of 50 100 mg/kg of L DOPA resulted in a dose dependant increase in fluorescence intensity per cell measured at the emission maximum for catecholamines The fluorophor resulting from the administration of L DOPA was indentified by spectral analysis of the emission showing a displacement of emission maximum of the mast cells towards shorter wavelengths characteristic for the catecholamine spectra and by analysis of the photodecomposition of the fluorophores In normal rats the mast cell fluorescence showed a rapid exponential fading similar to that of protein droplets containing 5 HT while the mast cells in DOPA treated rats showed a slower fading which appeared intermediate between that of 5 HT and dopamine-containing protein droplets

Fluorescence microscopy of various tissues rich in 5 HT containing mast cells showed a yellow fluorescence of the mast cells in control rats and in DOPA treated rats a blue green fluorescence approximating the blue fluorescence of catecholamine containing structures Biochemical analysis of catecholamines was performed on pooled rat tongues containing an abundance of mast cells In normal rats only traces of dopamine were found The injection of L DOPA resulted in an increase of dopamine but no change in noradrenalin concentration No traces of adrenaline were found

A Sima, Y Olsson & P Sourander EXPERIMENTAL OBSERVATIONS IN PERIPHERAL NERVES IN UNDERNOURISHED RATS

We have started a study of the effect of perinatal undernutrition on the sciatic nerve of the rat according to a method described by Chow & Lee 1964

The diameters of the nerve fibre population was studied lightmicroscopically in osmium fixed specimens and the frequency distribution was illustrated in histograms Only one peak was present (2 microns) in the controls up to 11 days of age Two peaks were not seen until rats were 17 days old corresponding to the adult pattern However in the undernourished rats up to 31 days of age there was only one peak at 1 5 microns This represented a higher proportion of the total number of counted fibres than the lower peak in control rats of the corresponding ages

The permeability of the perineurium was examined by injecting Evans blue albumin around the nerve In control animals a diffusion barrier to the labelled protein had developed in the perineurium from an age of about 3 weeks in the undernourished this had not occurred even at an age of 39 days

The results indicate that the growth of the nerve fibre calibres of the sciatic nerve is reduced in perinatally undernourished rats, presumably indicating a reduced myelination The undernutrition also influences the postnatal development of the barrier function of the perineurium

K Kristensson & Y Olsson AGE DEPENDANT VARIATIONS IN THE SPREAD OF EXOGENOUS PROTEINS IN PERIPHERAL NERVE

Protein tracers (albumin labelled with Evans blue and horseradish peroxidase) were injected around the sciatic nerves of adult and suckling mice In adult animals the perineurium constitutes diffusion barrier to the transfer of the proteins into the endoneurium In the suckling animals on the other hand the barrier function was not yet developed and the tracers could readily diffuse into the endoneurium through gaps between the perineurial cells In adult mice tight junctions between adjacent perineurial cells prevent intercellular diffusion of peroxidase

After injection of peroxidase into the gastrocnemius muscle of suckling mice small intramuscular nerve branches showed diffusion into the endoneurium and axonal uptake of this tracer into vesicular organelles The corresponding motoneurones in the spinal cord also contained the tracers in small cytoplasmic granules around the nucleus There

was no tracer in the surrounding neuropil or at any other level of the spinal cord These observa t ons show that proteins may be transported in axons in a retrograde d rection from the periphery all way up to the originating nerve cell body

J Modee B Itemark & B Robertson ULTRASTRUCTURE OF THE ALVEOLAR EPITHELIUM AND THE ALVEOLAR LINING LAYER IN PARAQUAT INDUCED EXPERIMENTAL RESPIRATORY DISTRESS

Electron microscopic studies of rat lung 24 and 48 hours after subcutaneous in ection of paraquat 35 mg/kg body weight) revealed interst tial oedema and necrosis of membraneous and granular pneumocytes The alveolar lining lay r as visualized by ruthenium staining was clearly diminished 24 hours after the injection of paraquat These structural lesions may explain the functional derangement of pulmonary surfactant which has been demonstrated in paraquat induced experimental respiratory distress

B Lagerlof VARIATIONS IN MEGAKARYOCYTE PLOIDY IN MYELOPROLIFERATIVE DISORDERS

In polycythemia vera (PCV) there is a great variation in megakaryocyte morphology but a characteristic find ng is the occurrence of hypertrophic megakaryocytes whereas in chronic granulocyt c leukemia (CGL) there is regularly an abundance of markedly hypotrophic megakaryocytes

Megakaryocytes in Feulgen stained smears of bone marrow were photographed in monochromatic l ght at 500 mμ and the light absorption of the nuclear image analysed in a half automatic photometer By analysing mature granulocytes on the same slides with the same procedure the DNA values of megakaryocytes could be related to di ploid values In control cases with normal thrombopoies s

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n the 8 n

t ons were encountered in the DNA histograms with cells up to the 64 n level In CGL there was a regular sh ft to lower ploidy values Most of cells in all cases were at the 8 n level and no cells were above the 16 n level

The great variat ons in the ploidy of megakaryocytes in PCV might reflect various degrees of response to some stimulus and m ght be consistent with a reactive proliferation whereas the very regular deviation from the normal DNA histograms show by the megakaryocytes from cases with CGL m ght correspond to a more uniform disturbance in cellular proliferation and reflect a true neoplastic proliferation of the megakaryocytes

B Sandstedt and *B Jereb* CLINICAL PATHOLOGICAL STUDY OF WILMS TUMOUR

The prognostic significance of tumour extent at time of operation upon survival was evaluated in a series of 208 children with Wilm s tumour (nephroblastoma) Microscopical sections derived from 90 of the patients were revived and the morphology of tumours and tumour size was also assessed in relation to survival All of the 10 children with tumours less than 5 cm in diameter are still alive (3 20 years) As regards the tumours, however there was no correlation between tumour size and survival period Well-differentiated tumours closely resembling embryonic kidney tissue were associated with a 2 year survival rate of 70 per cent The corresponding survival ra e for the most poorly differentiated tumours was 25 per cent. The results underline the prognostic importance of early diagnosis As to the small, well-differentiated tumours the prognosis of which is favourable surgery may be adequate and patients escape to be exposed to the risk of irradiation and chemotherapy

Th Berge & G Östberg BILATERAL BREAST CANCER

On the basis of conventional criteria, 82 cases of bilateral mammary carcinomas were diagnosed among 687 women with breast cancer autopsied during the 12 year period 1958 1969

Calculated on the basis of the survival time of all 687 subjects the expected number of a second breast tumour was 10 4 The frequency is thus about 8 times as high as in the normal population The risk of developing a second lesion was h gher before (11 times) than after (7 times) the menopause Most of the second cancers appeared within the next 5 year period but in one case it did not appear until 35 years later

C G Lindstrom THE FREQUENCY OF GALLSTONE DISEASE IN THE TOWN OF MALMÖ

In a necropsy series including 2 218 individuals over 20 years of age that is 88 2 per cent. of all deaths in these age groups in Malmo 1969 gall stones were found in 36,2 per cent of all cases, in 26 5 per cent of the men and in 46 8 per cent of the women

6 per cent. of the men and 11 6 per cent. of the women had been cholecystectomized Among those with gallstone-disease (gallstone at necropsy or postoperative state), 18 8 per cent. of the men and

20,2 per cent of the women had been subjected to cholecystectomy

Signs of gallstone-disease (gallstone at necropsy or postoperative state) were found in 44 per cent of all cases, in 31,9 per cent of the men, the frequency being highest—57,1 per cent—in the tenth decade and in 57 per cent of the women, the frequency being highest—63 per cent— in the eighth decade 10,9 per cent of the cases with postoperative state had stones in the remaining bile ducts

Chronic cholecystitis was found in 12,8 per cent of the men and 20,2 per cent of the women Acute cholecystitis was found in 0,9 per cent without any sex difference

Cancer of the gallbladder was found in 18 subjects, 4 men and 14 women Cancer of the bile ducts was found in 14 subjects, 6 men and 8 women

Peptic ulcers, scars or postoperative ulcer states were found in 17,9 per cent of all, in 20,9 per cent of the men and in 14,7 per cent of the women No statistical correlation between gallstone disease and peptic ulcer-disease could be proved

S Johansson, L Angervall, U Bengtsson and *L Wahlqvist* RENAL PELVIC CARCINOMA IN SWEDEN 1962-63

Earlier studies of renal pelvic carcinoma implicating a relationship between these tumours and long-term consumption of phenacetincontaining drugs have initiated a study of the national cases of renal pelvic carcinoma 1962 63 Preliminary results from a study of 86 of these cases hitherto available were presented

71 cases were operated upon and 15 of the cases had the tumours diagnosed at autopsy In the autopsy group there were 5 known abusers of phenacetin-containing drugs (1 man and 4 women) They had papillary necrosis and died from uraemia Sex ratio (man to woman) in the autopsy group was 1 2 and in the operated group 1,7 1 The high ratio of women seems to be explained by the higher frequency of female abusers

The 5-year survival rate in the operated group was 54 per cent for men and 32 per cent for women Among the operated cases 2 men and 4 women (8,5 per cent) were phenacetin abusers All of these had preexisting nephropathy with papillary necrosis

The prognosis was studied in relation to tumour size, infiltration depth, and histological grading 5 year survival declined with increasing tumour size decreasing differentiation and increasing infiltration of the tumour There was a tendency toward a lower differentiation of the tumour among the women

Multiple localized tumours to develop simultaneously or later were found in about 30 per cent of the operated patients

19 of the operated cases have been autopsied 8 of these had local recurrence (42 per cent), and 6 (32 per cent) showed lymph node metastases

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ENDOMETRIAL ENZYMATIC DEFECTS IN STERILITY

A Histochemical Study

HENNING JENSEN, GUDIK SORENSEN and HENNING PEDERSEN

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The activities of alkaline and acid phosphatases and of dehydrogenases (NADH, LDH, SDH, α-glyc and G 6 DH) have been studied in a material of endometrial biopsy specimens taken about six days after the ovulation from 23 women with undesired childlessness The results are in fair agreement with those of previous examinations of the endometrium, except regarding the activity of G-6 DH, which varied considerably In six women with no detectable cause of sterility no activity of G-6 DH could be demonstrated The possibility of a causative relation between blocking of the pentose shunt and sterility is suggested

Various reports on investigations into the endometrial contents of RNA, glycogen, mucin, and hydrolytic and oxidative enzymes are available (4) (11) (12) The varying enzyme activity during the menstrual cycle has likewise been described (6) (7) (8) (9) (11) (12) (13) (22)

The object of the present study was to investigate whether sterility, in the absence of a diagnosed organic cause is associated with abnormalities of the endometrial enzyme activity

MATERIAL AND METHOD

The series investigated comprised 23 women, who had all attended for examination at the Fertility Clinic Rigshospitalet (University Hospital) owing to involuntary childlessness for more than 18 months

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The present study is concerned solely with endometrial biopsies from women who are childless for different reasons No normal series has been included

A normal series must consist exclusively of women who are in *perfect health* at the time of investigation, and who have had no previous illness recorded, especially no disease of the pelvic region or the endocrine system The woman must have been delivered normally The hormone analyses *must* give normal values, and the basal temperature curve must show normal cyclic variations Further, the biopsy specimens must be taken at the

pensed with, biopsy specimens being obtained from

e that tissue of histologically normal appearance from patients subjected to abrasion on account of menstrual irregularities *The* authors of the present paper regard normal materials encumbered with the above uncertainty factors as unsuitable

The patients ranged *in* age from 22 to 38 Half were between 28 and 30

All the patients were subjected to *gynaecological* examination in addition to ordinary clinical examination In relation to the former, two *endometrial* biopsy specimens were taken using Reiffenscheid s *curette* *The patients* were examined as out patients and no anaesthesia was given About 10 minutes elapsed from the specimen was taken till it was frozen down

Basal temperature curves were plotted for all the patients, *and the endometrial* biopsy specimens were preferably taken six *days after the ovulation* reckoned on the basis of the temperature curve

The pregnanediol excretion was *measured* by the *time the biopsy* specimen was removed In addition the *patient s excretion of 17* ketosteroids and 17 ketogenic *steroids were measured* and finally the pituitary gonadotropin excretion *The patients* husbands had their sperm examined at least twice

The two tissue specimens from each patient were treated differently *One* was fixed in 86% *alcohol* and the other was rapidly frozen down at — 80°C *in an isopentane/acetone/carbon* dioxide dry ice freezing mixture, thereafter to be stored at *— 20° C*

The alcohol fixed tissue specimens were embedded *in paraffin*, and the sections produced were *stained with* haematoxylin eosin and according to van Gieson Hansen

These preparations were used for ordinary *histo*logical description and phase evaluation

The frozen tissue specimens were cut on cryostat (Pearse SLEE) in about 8 μ thick sections Staining *tests were* made for nicotinadenine dinucleotide diaphorase (NADH) lactic acid *dehydroge*nase (LDH) succinic acid dehydrogenase (SDH) glucose 6 phosphate dehydrogenase (G 6 DH), and alphaglycerophosphate dehydrogenase (a glyc) The staining *method* employed was that indicated by *Pearse (20)* The tetrazolium salt nitro BT was used as indicator

The activities of the alkaline and acid phosphatases were likewise studied The former were demonstrated by Pearse s method (21) *using α naph*thylphosphate as substrate and Fast red TR as indicator The sections were contrast *stained with* haematoxylin For the acid phosphatases α naphthylphosphate was likewise used as substrate The indicator was pararosaniline and the sections were contrast stained with methyl green *Barka (2) has* introduced this method

All the tissue specimens were incubated for 20 minutes The temperature was 38° C for the de*hydrogenases and 20° C* for the phosphatases

At the reading of the enzyme activities a semi quantitative assessment was aimed at, based on an activity scale from 0 to 4 Grade 0 represented no activity grade 1 slight activity (the tissue was only stained just enough to make the glandular structures distinguishable) while grade 2 indicated moderate activity and grade 3 strong activity (marked reaction with distinct delineation of the tissue structures but with no over staining) *Grade 4* indicated very strong reaction with overstaining *of the tissues*

RESULTS

NADH strong reaction of the glandular epithelium with an even distribution in the cytoplasm though with a slight tendency towards luminal accumulation of the *reaction pro*duct The intensity varied little from gland to gland, and the cells within the individual glands showed uniform activity

In 21 preparations the glandular activity was of grade 3 and in two of grade 2 The stromal reaction averaged grade 1 2 with diffuse distribution of the reaction product *It was often difficult* to distinguish the vessels from the surrounding stroma by the dehydrogenase stainings the reaction of vessels and stroma being of equal intensity

LDH The same sites and almost the same intensities of reaction as NADH Fourteen preparations showed activity of grade 3 and nine of grade 2

A characteristic feature of all the dehydrogenases was that they reacted in the same region of the glandular epithelium but at different intensities In the stroma NADH and LDH displayed weak activity, while the three other dehydrogenases tested gave *no* reaction in this tissue

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Fig 1 Glukose 6 phosphate dehydrogenase Magnification × 100 Incubation period 20 minutes Abundant *formazan granules are found throughout* the cytoplasm of the epithelium as an indication of *intense activity Prep no 91 69*

Fig 2 Glukose 6 phosphate dehydrogenase Magnification × *100* Incubation period *20 minutes* Note the lack of staining as an indication of *no activity* *Prep no 500/69*

Fig 3 NADH *diaforase* Magnification × 100 Incubation period 20 minutes Note the heavy activity diffuse in the cytoplasm of the epithelium Prep no 500/69

Fig 4 Acid phosphatase Magnification × 100 Incubation period 20 minutes The reaction products are localized in the luminal zone of the epithelium No activity in the surrounding stroma Prep no 500/69



2



3



SDH little activity in the material under review Fourteen preparations did not react at all In eight the *intensity of reaction* was of grade 1, and in no more than one of grade 2

G 6-DH showed the greatest range of variation Ten preparations did not react In six the intensity was of grade 1, in five of grade 2, and in two of grade 3

α-glyc activity absent *in ten preparations,* while in five it was of grade 1 and in two of grade 2 In six cases no staining was performed

The alkaline phosphatases showed in all the preparations a strong reaction localized in the vessels In general the stroma displayed no activity beyond that relating to the vessels However, in a small number of preparations isolated cells with strong activity (presumably leucocytes) were found in the stroma The activity was localized mainly in the glandular epithelium The reaction product was found diffusely in the cytoplasm, with a tendency towards luminal accumulation In some preparations a basal reaction band was also seen, which effected development of a double layer zone The nuclei did not react The intensity varied considerably from one site to another Thus, the individual fields might present both intensely reacting and totally non reacting glands Differences from cell to cell were seen even within the same gland Among the 25 preparations studied, one was a failure Four showed no reaction nine activity of grade 1, four of grade 2, and five of grade 3 There were no over-stained preparations

The acid phosphatases were likewise localized in the glandular epithelium with a tendency towards luminal accumulation of the reaction product The nuclei were not stained The reaction varied from gland to gland, though less than in the case of the alkaline phosphatases The stroma displayed slight, diffuse activity of grade 0–1 The vessels reacted with the same intensity, being accordingly difficult to identify In the stroma there were often found isolated, strongly reacting cells, presumably macrophages No tissue specimens were completely without activity In three the intensity was of *grade 1, in five of* grade 2, and in nine of grade 3 Six preparations were over-stained and estimated at grade 4

DISCUSSION

Previous Investigations

The normal intensities of the various enzymes in the menstrual cycle have been reported in the literature

NADH shows low activity in the proliferative phase, with a rise at the beginning of the secretory phase The activity thereafter remains high throughout the rest of the cycle (6) (4)

LDH shows low activity during the proliferative phase and a rising intensity during the early secretory phase This is followed by constantly high values during the rest of the cycle (4)

Some disagreement prevails regarding the activity of SDH in the endometrium *Cohen et al* (6) state that SDH shows a constant weak activity throughout the cycle *Filipe et al* (7) likewise found no cyclic variation of the activity In the present material of endometrial biopsy specimens taken during the secretory phase the reaction was likewise weak Others have found a low activity at the beginning of the proliferative phase followed by a rise to high values close to the time of ovulation (8) The intensity has been claimed to remain high until about the 21st day thereafter to fall to low values (4) (15) A few investigators have noticed high activity until the 24th day *Stuermer* (22) states that despite the fall in the activity by the end of the secretory phase the activity is *still higher* than that at the beginning of the proliferative phase

The enzymes of the tricarboxyl acid cycle have been thoroughly investigated, whereas the enzymes of the pentose shunt have not been studied *Only few investigations into the G 6 DH activities in the endometrium are* available

Some workers (11) (7) claim that G 6 DH shows constant activity throughout the cycle

with no correlation to the glycogen deposit ing *Filipe et al* (7) arrived at similar values by qualitative estimation whereas quantitative estimation gave a rise in the G 6 DH activity late in the secretory phase *Hughes et al* (11) mention that the activity of this enzyme in the endometrium seems to be independent of hormonal influence This harmonizes badly with the activity and hormone dependence of G 6 DH in other organs In thyrotoxicosis the erythrocytes display high activity of G 6-DH A correspondingly low value is found in myxoedema (23) The postmenopausal endometrium displays little activity of G 6 DH but this becomes greatly increased by treatment with oestrogens (7) *Mori et al* (17) (18) found tumours in organs with an endocrine function to present a high activity of G 6-DH (thyroid body suprarenals ovaries) On the whole the enzyme thus shows pronounced hormonal dependence Further variations may be found in other conditions Thus in pernicious anaemia an increased activity is seen in the erythrocytes of man (5) Experimentally provoked B avitaminosis in rats has resulted in low values in the liver (3) and castration – likewise of rats – has been found to cause alterations of the G 6 DH activity in the liver (10) Haemolytic anaemia involves increased activity in the erythrocytes (5)

The alkaline phosphatases react strongly during the proliferative phase with high values about the time of ovulation The values remain high until the 21st–22nd day after which a steep fall is seen (4) (7) (9) (10) (11) (12) (15) (16) The enzyme is hormone-dependent (7) (14)

The acid phosphatases react weakly during the proliferative phase and strongly during the secretory (9) (15) with maximum just before menstruation (4) (12) The enzyme is subject to hormonal dependence as stated by Kvarstein (14) among others who found a rising activity of acid phosphatases after treatment with the progesterone preparation quingestanol

The activity of α glyc in the endometrium seems not to have been studied previously The enzyme catalyzes the oxidation of L-α-glycerophosphate to dihydroxyacetone, which is of importance in the synthesis of neutral lipids and phospholipids (19)

It has been claimed that the following alterations may take place in the endometrium in relation to sterility and habitual abortion Low glycogen concentration (24) (11), unchanged activity of NADH and LDH (11), and varying often low values of alkaline phosphatases and RNA (11)

Discussion of Author's Results

The present study was carried out with a view to investigating whether it might be possible by histochemical methods to detect alterations in the endometrium serviceable as a means of grouping a series of sterile women

As some of these patients presumably were normal we attempted to single out this group and employ it as a possibly normal series

All the subjects examined have been included in *Table 1*

The women grouped above the upper, horizontal, dotted line are those supposed to be normal Clinically they displayed no abnormality and the hormone analyses gave normal results As regards case no 668/69 we have information of previous abortion while nr 280/69 is known previously to have carried through a normal pregnancy The patient whose pregnancy led to abortion may with some justice be included in the group of possibly normal , as it was not a question of habitual abortion and the enzyme activities corresponded to the normal values reported in previous publications

The next group (that between the two horizontal dotted lines) comprises 12 patients who presented changes that might be responsible for the sterility These were women with congenital anomalies sequelae of inflammatory states and abnormal hormone analyses Further, the group included one patient who previously had been subjected to subtotal strumectomy owing to thyrotoxicosis and four cases of secondary sterility (One of the latter patients had previously carried

TABLE 1 *Table Giving a Survey of the Examined Patients*

	Age	NADH	LDH	SDH	G 6–DH	α glyc	Alc phosph	Acid phosph	Male fert	Hormone analyses	Pregn prev or pres	Prev diseases	Present diseases
668/69	27	3	3	2	2	1	1	3	reduced	norm	ab	none	none
652/69	32	3	3	1	2	2	3	4	reduced	norm	—	none	none
280/69	37	3	2	1	1	1	1	2	reduced	norm	partus	none	none
592/69	31	3	3	0	1	0	2	3	reduced	norm	—	none	none
16/69	29	3	3	0	2	0	0	1	reduced	norm	—	none	none
78/69	29	3	3	0	3	1	0	3	reduced	norm	—	strumec f thyrotox	
504/69	26	3	3	1	1	1	1	3	asperm	norm	—	bicorn uterus	
223/69	33	3	2	0	2	2	3	4	reduced	norm	ab	op f retroflex uteri	
1003/68	23	2	2	0	0	0	3	2	reduced	norm	ab	op f retroflex uteri	
94/69	28	3	3	0	3	0	1	3	reduced	norm	—	bilat tubal occl	
515/69	29	3	3	1	0	0	1	2	norm	abn	partus	bilat tubal occl	
784/69	30	3	2	1	1	–	1	3	norm	norm		op extraut pregn + fibr ut	
625/69	30	3	2	1	1	–	2	4	reduced	norm	—	none	ob fibr ut
519/69	26	3	2	1	1	–	3	2	norm	norm		salpingit 1969	none
1086/69	26	3	3	0	0	0	1	2	reduced	norm		salpingitis	none
626/69	28	3	3	1	2	–	3	3	reduced	abn ?		none	oligomenorrh
272/69	30	3	2	0	0	–	–	4	norm	abn		Stein Leventhal's syndr	
1025/68	28	3	2	0	0	0	0	1	asperm	norm		none	none
1036/68	29	2	2	0	0	0	0	3	reduced	norm		none	none
1044/68	36	2	3	0	0	0	1	4	reduced	norm		none	none
143/69	38	3	3	0	0	1	3	1	reduced	norm		none	irreg cycle
500/69	28	3	3	0	0	0	1	3	norm	norm		none	none
510/69	27	3	3	0	0		2	4	reduced	norm		none	none

Above the possibly normal group In the middle between the two horizontal dotted lines patients with morbid conditions which may account for the sterility Below a group of patients with no clinical signs or symptoms but presenting an abnormal enzyme histochemical structure (failing G 6 DH activity)

through a normal pregnancy while the others miscarried)

The group is inhomogeneous a fact also reflected in the results of the enzyme histochemical tests

The lower group comprising six women is characteristic in that the women are clinically normal with normal results of hormone analyses The enzyme histochemical tests showed failing activity of G-6 DH in all the specimens Thus, these subjects constituted a group of sterile women whose only established defect was found in the endometrial enzyme system

It is shown in the table that *NADH* and *LDH* presented high values in all three groups corresponding to the values stated in the literature

The activity of *SDH* was low in most cases The short incubation period employed was an essential cause of this Such a period was preferable because it afforded a possibility of

comparing the intensities of the various dehydrogenases all incubated for 20 minutes If a longer incubation period was employed, other dehydrogenases e g LDH would become over stained and in many cases useless

The results regarding the staining reactions of *α* glyc showed the highest values in the possibly normal group

The alkaline phosphatases displayed varying activity in all three groups with no specific alterations

The acid phosphatases likewise presented varying activity in relation to certain groups in the table

G 6 DH The middle group comprised four patients with failing G 6-DH activity 1086/69 had normal hormone analyses but she had previously been affected with salpingitis We do not yet know for certain whether inflammatory states may cause permanent reduction of the G 6 DH activity Nos 223/69 and 1003 69 had previously miscarried 515/69 who had previously carried through a normal pregnancy presented abnormal hormone values at the time of the time of the biopsy 519 69 is stated to have had an attack of salpingitis six months before the present investigation 1086/69 is likewise known to have had salpingitis 78/69 had been subjected to partial strumectomy owing to thyrotoxicosis She was euthyroid at the time of the investigation As alterations of the thyroid function influenced the activity of G 6 DH this patient had to be referred to the middle group though the G 6 DH activity was high in this case

In *the lower group* of the table no clinical or anamnestic data were available that might account for the sterility All the biopsy specimens were characterized by total absence of G 6-DH activity SDH showed likewise no activity However no definite importance can be attached to this fact because this enzyme displayed slight or no activity *in all the other* biopsy specimens except 668/69 The same was the case with *α* glyc

The activities of G-6-DH in the three groups differed so much in *intensity* that the possibility of a defect of the pentose shunt should be considered

A possible blocking of the pentose shunt will confound the RNA synthesis The low endometrial concentration of RNA in cases of sterility and habitual abortion as described by *Hughes et al* (6), can thus be explained by failing G 6 DH activity Finally, there is reason to suppose that repeated endometrial biopsies carried out before and after a possible hormone treatment, would show variations of the G 6-DH activity, if the treatment had any effect The present material was not subjected to such investigations, which however, would probably be of interest from a therapeutic aspect

CONCLUSION

In the material under review, comprising endometrial biopsy specimens taken, within the secretory phase, from sterile women we may perhaps be justified in singling out a special group of women with no clinically detectable cause of sterility but who all presented failing activity of G 6-DH

The possibility of a causal relation between blocking of the pentose shunt with a consequent reduced RNA synthesis and sterility exists

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STROMAL RESPONSE IN BREAST CARCINOMA AND FIBROADENOMATOSIS, ESTIMATED BY THE AID OF THE ALKALINE PHOSPHATASE ACTIVITY

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80 specimens of mammary tissue presenting benign and malignant changes were subjected to biopsy with a view to disclosing activity of unspecific alkaline phosphatases The material comprised 40 carcinomas 32 cases of fibroadenomatosis 7 fibroadenomas, and 1 carcinoma in situ In the cases of carcinoma no or only slight phosphatase activity was found in the tumour cells The surrounding stroma often showed 'reaction zones' formed by proliferating fibroblasts with high phosphatase activity This stromal response was found in 65 per cent of the biopsy specimens of carcinoma, while another 7 5 per cent were doubtfully positive The incidence of reaction zones was seen to increase appreciably with rising grades of tumour anaplasia (Grade I 33 3 per cent, grade II 65 per cent, and grade III 90 9 per cent) Further, correlation was found between reaction zones and stromal lymphocyte infiltrates. The pathogenesis of the reaction zones is discussed and the conclusion has been drawn that a stromal reaction against the tumour must be the most likely hypothesis In fibroadenomatosis 18 8 per cent of the biopsies revealed reaction zones The question has been considered whether the observation might be employed for a delimitation of a high risk group of patients with fibroadenomatosis

Reports on enzyme-histochemical analyses of breast cancer are comparatively rare in the otherwise comprehensive literature on histopathological biochemical, and — within recent years — electron microscopical examinations of mammary tissue Reports concerned with studies on the enzyme activities in the stroma surrounding the tumour tissue are even rarer

In the present paper an account will be given of an investigation into the stromal response, estimated by the aid of the activity of the unspecific alkaline phosphatases. The results have been compared with the intensities in tissue sections of fibroadenomatosis

MATERIAL AND METHODS

The investigation was based on 80 biopsy specimens of mammary tissue from women referred to the Surgical Departments of Rigshospitalet (University Hospital) with a diagnosis of breast tumour The material comprises 40 cases of breast carcinoma 32 with fibroadenomatosis, 7 fibroadenomas and 1 case of ductal carcinoma in situ

The tissue specimens, taken during the operation, were immediately frozen in an isopentane/acetone/dry ice (solid CO_2) at —80°

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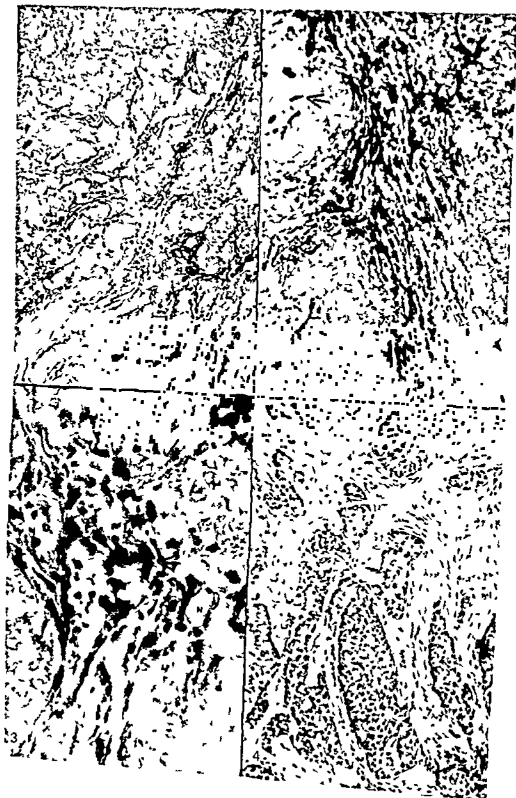
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TABLE 1 *Relation between the Incidence of Reaction Zones and Grade of Anaplasia*

	+ reaction zones	— reaction zones	? reaction zones	Total
Carcinomas				
grade I	3 (33 3 %)	4 (44 5 %)	2 (22 2 %)	9 (22 %)
grade II	13 (65 0 %)	6 (30 0 %)	1 (5 0 %)	20 (50 %)
grade III	10 (90 9 %)	1 (9 1 %)	0	11 (28 %)
	26 (65 0 %)	11 (27 5 %)	3 (7 5 %)	
Fibroadenomatoses	6 (18 8 %)	25 (78 1 %)	1 (3 1 %)	32
Fibroadenomas	0	6 (85 7 %)	1 (14 3 %)	7
Carcinoma in situ	1	0	0	1
				Total 80

Fresh frozen tissue specimens, about 8 μ thick were cut on cryostat (SLEE Pearse) *Pearse s* (21) method was employed for detecting the activity of unspecific alkaline phosphatases Alpha naphthyl phosphate was used as a specific substrate, and Fast red TR as indicator The tissue specimens were contrast stained with haematoxylin The incubation period was 20 minutes at 20°

In addition to the phosphatase test stainings fresh frozen sections and paraffin sections were subjected to staining with haematoxylin and eosin

The following procedure was employed in the assessment of the preparations All the tissue sections stained for the purpose of disclosing alkaline phosphatase were assessed by one of us (H J) The preparations were examined at least twice at intervals of some months without the examiner knowing the results of previous tests

The other (T S) examined the tissue sections stained with haematoxylin and eosin and grouped the carcinomas according to grade of anaplasia on the basis of the system indicated by *Bloom & Richardson* (3) The tissue specimens presenting fibroadenomatosis were first classified according to *Kiær s* (13) method Such a graduation proved however to be unfit for the present material In many cases the relatively small size of the biopsy specimen (about ½ × ¾ × 1 cm) precluded a reliable graduation We therefore chose to collect all the cases of fibroadenomatosis in one group

After the conclusion of the series of tests the results were compared and set out in Tables 1 and 2

RESULTS

Responses in Carcinoma

A total of 40 breast carcinomas were studied Seven displayed slight to moderate activity of alkaline phosphatases in the tumour cells, while 33 showed no activity whatever The endothelium of the vessels showed high activity in all the preparations thus demonstrating the usefulness of the staining method for studying the vascular structure of the carcinomas

In the preparations which in addition to tumour tissue, contained ductules this presented the same enzyme activities as those described in the section on fibroadenomatosis

The stroma surrounding the tumour was often found to contain an increased number of fibroblasts, grouped in islands and bands tending to form concentric structures round

Fig 1 Alk phosph Magnif × 70 Carcinoma with reaction zones in the surrounding stroma The phosphatase activity is most intense in the right side of the field of vision Above to the left no reaction zones are seen The tumour cells show no activity

Fig 2 Alk phosph Magnif × 280 Islands of tumour tissue with reaction zones consisting of phosphatase positive fibroblasts The vessels (marked with arrows) also show activity

Fig 3 Alk phosph Magnif × 700 Same preparation as Fig 2 The vessels (marked with arrows) are distinct from the fibroblast

Fig 4 Magnif × 100 Carcinoma without reaction zones Many fibroblasts are seen but no phosphatase activity

eaction Zones and Lymphocyte Infiltrates

	Fibroadenomatosis			Fibroadenoma		
— act zones	+ react zones	? react zones	— react zones	+ react zones	? react zones	— react zones
6 (37.5 %)	4 (13.9 %)	1 (3.4 %)	24 (82.7 %)	0	1	6
3 (25 %)	0	1	1 (7 %)	0	0	0
2 (25 %)	0	0	0	0	0	0
0	1	0	0	0	0	0
11	5	2	25	0	1	6

were found to be of the same appearance as that described for the carcinomas (Fig 8) A single specimen presented diffusely scattered phosphatase positive fibroblasts all over the stroma This specimen was characterized as having a doubtfully positive reaction zone (see Table 1)

Fibroadenomas

The activity in the epithelium corresponded to that noticed in fibroadenomatosis, except that it was less pronounced The vascular activity was distinct No reaction zones were found in the stroma but a single biopsy specimen contained diffusely scattered phosphatase positive fibroblasts, being therefore referred to the group characterized as having doubtfully positive reaction zones

Most often many fibroblasts were found in the stroma but these displayed no phosphatase activity

DISCUSSION

The enzyme histochemical structure of breast carcinoma and fibroadenomatosis has been described previously The results often enter into studies comprising both hydrolytic and oxidative enzymes (8) (12) (15) (20), while others are only concerned with the activity of hydrolytic enzymes (2) (5) (6) (9) (14)

In the material under review most of the biopsies revealed failing activity of alkaline phosphatases in the tumour cells, while a minor proportion showed slight to moderate activity This is in agreement with the statements in previous publications

Only few descriptions have been given so far of the stromal response (10) (16) (17) To our knowledge, *Monis et al's* (16) and *Murata et al's* (17) publications are the only ones in which investigations into the stromal response have been reported, considered in relation to the grade of anaplasia of the tumour Neither does the occurrence of reaction zones in fibroadenomatosis seem to have been described

A review of the literature revealed some publications in which the illustrations and their attending legends show that phosphatase positive fibroblasts may occur in the stroma round the tumour in cases of breast carcinoma (8) (20) (6) Though the subject is only touched on sporadically in these publications, it may with some justification be claimed to bear out the results of the present study

The works by *Huseby et al*, *Monis et al*, and *Murata et al* will be described in greater detail, their results having been of importance in the evaluation of the present investigation

TABLE 2 *Relation between the Inc d n*

Intensity of lympho cyte in filtr	Number of biopsy specimens	All biopsy specimens			Carcinoma	
		+ react zones	? react zones	— react zones	+ react zones	? react zones
0	53	12 (22 6 %)	4 (7 6 %)	37 (69 8 %)	8 (50 %)	2 (12 5 %)
+	14	8 (57 1 %)	2 (14 3 %)	4 (28 6 %)	8 (66 7 %)	1 (8 3 %)
+ +	8	6 (75 %)	0	2 (25 %)	6 (75 %)	0
+ + +	5	5 (100 %)	0	0	4 (100 %)	0
	80	31	6	43	26	3

the tumour tissue These fibroblasts presented pronounced activity of unspecific alkaline phosphatases Below these structures will be termed reaction zones In some cases fibro blasts were also seen spread diffusely in the stroma but in such cases only slight or no phosphatase activity was detectable

In typical cases the reaction zones formed as stated, concentric bands in the stroma round the tumour Often however smaller bands were seen to pass in between the indi vidual islets of tumour tissue The latter thereby became divided up by a phosphatase positive network (Fig 1 2 3)

As seen in Table 1 reaction zones were definitely present in 65 per cent of the exam ined carcinomas while another 7 5 per cent were doubtfully positive By relating the in cidence of reaction zones to the grade of anaplasia of the tumours we noticed a mark ed increase of the number of biopsy speci mens with reaction zones with rising anapla sia grades (from 33 per cent at grade I to 90 per cent at grade III)

An attempt was made to throw some light on occurrence of inflammation in connection with reaction zones by relating the intensities of possible lymphocyte infiltrates to the in cidence of reaction zones The result is seen in Table 2, which shows a tendency towards a more frequent occurrence of reaction zones parallel with a rising intensity of the lympho cyte infiltrates

Responses in Fibroadenomatosis

Biopsy specimens from 32 cases of fibro-adenomatosis were studied The reaction to unspecific alkaline phosphatases showed pro nounced activity in the basal parts of the ductules localized to the myoepithelial cells In addition many preparations showed phos phatase activity luminally in the epithelium resulting in formation of a doublelayer struc ture in the epithelium (Fig 6)

Cystic formations when present generally showed somewhat weaker activity than the ductules (Fig 7)

In cases with intraductal epithelial pro-liferation phosphatase activity was seen at the site of the myoepithelial cells whereas the proliferating epithelium itself showed no activity In a minor proportion of the cases however the stained phosphatase reaction product was seen to extend up through the whole epithelial layer

The vascular activity was pronounced and strong in all the preparations Varying num bers of fibroblasts were found in the inter and intralobular tissue These generally did not react or showed only slight phosphatase activity In 18 per cent of the biopsy speci mens with fibroadenomatosis reaction zones

Husby et al (10) studied the activity of unspecific alkaline phosphatases in inoperable breast tumours before and after hormone treatment They noticed frequent occurrence of phosphatase positive fibroblasts in the surrounding tissue after the treatment and attributed this increased activity to the hormones pointing out that no similar activity was found in untreated carcinomas

Monis et al (16) examined 87 benign and malignant tumours among which nine were breast tumours They described fibroblast proliferation and phosphatase activity as frequent findings and concluded that the increased enzyme activity was due to inflammation, whereas not to the tumour's presence in the tissue In the stroma, *Monis et al* (16) noticed phosphatase activity in fibroblasts collagenous fibres, and polymorphonuclear leucocytes, in addition to the activity always present in the endothelium of the vessels Further, there is reason to mention the following observations reported in the stated paper 1) The intensity of the phosphatase activity varied considerably in most of the tumours 2) No correlation was found between the phosphatase activity in the stroma and invasive growth nor between the increased enzyme activity and the grade of anaplasia of the tumour 3) Benign tumours and chronic inflammatory processes showed a high phosphatase activity

Murate et al (17) studied the activity of unspecific alkaline phosphatase in 409 malignant and 225 benign tumours, among which 35 were breast carcinomas and 69 tissue specimens with fibroadenomatosis The stroma surrounding benign tumours displayed only slight activity The stroma surrounding malignant tumours was subject to inflammation, and accordingly showed activity of alkaline phosphatases It is emphasized that no correlation was found between the severity of the inflammation and the intensity of the phosphatase activity (In other words, disagreement with the observations of *Monis et al*) Carcinomas developed in a chronic gastric ulcer were pointed out as an example In such cases massive inflammation was found, but no phosphatase activity *Murate et el* mentioned various factors as possible causes of the phosphatase activity among which hormonal action inflammation, and a specific action from the tumour

The cause of the formation of the characteristic reaction zones has not been clarified

Below, an attempt will be made to assess the results of the present study on the basis of the three stated hypotheses regarding the cause of the increased phosphatase activity 1) hormonal action 2) inflammation, and 3) specific reaction to the tumour

Theory 1

Hormonal action as the cause of increased activity of alkaline phosphatases in murine breast cancer has been mentioned by *Richardson et al* (22) among others They used oestrogen in their experiments *Fanger et al* (6) claimed that the activity of alkaline phosphatases in normal mammary tissue increases during oestrogen treatment

Husby et al (10) were likewise of the opinion that the stromal response is due to the action of oestrogens Their results are, however, in disagreement with those of more recent investigations as they found no phos

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Fig 5 Alk phosph Magnif × 100 Carcinoma without reaction zones Vessels with phosphatase activity are seen (most pronounced in the right lower part of the field of vision marked with arrows The fibroblast show no activity

Fig 6 Alk phosph Magnif × 280 Fibroadenomatosis Phosphatase activity localized to the myoepithelium and in a few regions luminally in the epithelium The vessels likewise show activity whereas not so the fibroblasts

Fig 7 Alk phosph Magnif × 70 Fibroadenomatosis Activity localized to the myoepithelium and the vessels The proliferating epithelium and the fibroblasts show no activity

Fig 8 Alk phosph Magnif × 350 Fibroadenomatosis with reaction zones of phosphatase positive fibroblasts situated in concentric bands round a fairly large excretory duct A vessel likewise showing activity is seen lowest to the left in the field of vision

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8

admixture of a little of the surrounding stroma He regarded this observation as a proof of an interaction of tumour cells and surrounding stroma

We incline to support theory no 3

CONCLUSION

The following results of the present study substantiate our view that the reaction zones represent a specific reaction to the tumour tissue

1) A significant difference between fibroadenomatosis and carcinomas regarding the incidence of reaction zones
2) Increase of reaction zones with rising grades of anaplasia
3) A tendency towards an increase of reaction zones with rising lymphocyte infiltration

A consequence of the above hypothesis would then be that the 18 8 per cent biopsy specimens with fibroadenomatosis presenting reaction zones should be regarded as a high risk group representing the patients who might subsequently develop carcinoma To draw definite conclusions a larger material is however required (Such a material is being studied) Especially it is unfortunate that the present material includes only a single carcinoma *in situ* Further there is reason to point out that the observation period for the examined patients did not exceed two years so that accordingly the prognostic value of the reaction zones cannot yet be assessed

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phatase activity in the stroma surrounding the non treated tumours *Murata et al* (17), for instance, observed stromal response with phosphatase activity in about 80 *per cent* of the examined malignant tumours

We cannot subscribe to the theory of *hormonal action* as the cause of reaction zones Thus for instance, the fact that *no preponderance* of reaction zones is *found in fibroadenomatosis* argues against this hypothesis *Such preponderance* might be expected from the hypothesis of an influence of hormonal *factors* on the development of fibroadenomatosis *Robbins* (23) has written about this that it is now almost universally accepted that it results from an exaggeration and distorsion of the cyclic breast changes that normally occur in the menstrual cycle

If hormonal factors had any influence on the occurrence of reaction zones we should expect to find accumulation of reaction zones in cases of fibroadenomatosis This was not so in the material under review

Theory 2

Monis et al (16) concluded that the occurrence of stromal response to phosphatase activity is due to inflammation They noticed high activity in existing polymorphonuclear leucocytes in unspecific granulation tissue, and in chronic inflammatory processes

A high phosphatase activity is always seen in the presence of infectious inflammatory infiltrates as also observed by us in mammary tissue with abscess formations (These biopsy specimens were not included in the material)

Monis et al (16) also found a high phosphatase activity in the actively proliferating fibroblasts that may be present in fibroadenomas This observation is in disagreement with that of *Murata et al* (7) and with the results of the present study

In an attempt to estimate the relation between possible inflammatory infiltrates and the incidence of reaction zones we determined the intensity of lymphocyte infiltration visible in the stroma round the tumours in *the present material* The results have been set out in Table 2 The incidence of *reaction zones* was calculated — partly for all *the biopsy* specimens, and partly for carcinomas and fibroadenomatoses separately We regard the material as too small to allow of *conclusions* (For the same reason no statistical calculations have been performed) The results suggest, however, a *tendency* towards a percentage rise of the number of reaction zones with *increasing density* of lymphocyte infiltrates The cause of occurrence of these mononuclear infiltrates has not been definitely clarified To day the hypothesis most commonly inclined to is that of an immunoreaction to the tumour tissue (1) (4) (7) Absence of polymorphonuclear leucocytes renders an infectious aetiology less likely The prognostic value of the lymphocyte infiltrations is doubtful However, *Schiodt* (24) *found a significantly* prolonged survival in the group with breast carcinomas of grade III, if massive lymphocyte infiltrates were seen at the same time in the surrounding stroma

Murata et al (17) found no unquestionable correlation between the inflammatory response of the tumour and the phosphatase activity However, it is not plain to see from their material whether infectious infiltrates (polymorphonuclear leucocytes) are included in the concept of inflammatory response Accordingly their material is not comparable with the present (Table 2) which solely includes the intensity of lymphocyte infiltrates

Theory 3 — Specific Reaction to the Tumour

Murata et al (17) mentioned this hypothesis *Oka et al* s (18) observations of stromal response by the aid of phosphatase activity without concurrent inflammatory infiltrations (in planocellular carcinomas) likewise support this hypothesis Finally mention should be made of *Iwamori's in vitro* experiments (11) in which he demonstrated interaction of tumour cells and surrounding fibroblasts He pointed out the difficulty of culturing tumour cells without simultaneous

TABLE 1 *Diagnostic Procedures*

Case no	Clinical development	Prim	Res	Blind Test A	B	C	D	F	F	Sen	Jun	Vote B	Vote M	Cumulative
1	Malign †	M	M	B	M	M	M	M	M	M	M	1	9	M
2 NB	Malign †	B	B	M	B	B	B	B	B	B	B	9	1	B
3 NB	Benign	M	B	B	M	M	M	M	B	B	M	4	6	M
4	Benign	B	B	B	B	B	M	B	M	B	B	8	2	B
5	Malign †	M	M	M	M	M	M	M	B	M	B	2	8	M
6	Malign †	M	M	M	M	M	M	M	B	M	M	1	9	M
7	Malign †	M	M	B	M	M	M	M	B	M	M	2	8	M
8	Benign	M	B	B	B	B	B	B	M	B	B	8	2	B
9 NB	Benign	M	M	M	M	M	M	M	M	M	M	0	10	M
10	Benign	B	B	M	B	B	B	B	B	B	B	9	1	B
11	Malign *	M	M	M	M	M	M	M	M	M	M	0	10	M
12	Benign	M	B	B	B	M	B	B	B	B	M	7	3	B
13	Benign	B	B	B	B	M	M	B	M	B	M	6	4	B
14	Benign	B	B	B	B	B	B	B	B	B	B	10	0	B
15	Benign	M	B	B	B	M	B	B	B	B	B	8	2	B
16	Malign †	M	M	M	M	M	M	M	B	M	M	1	9	M
17	Malign †	M	M	M	M	M	M	M	B	M	M	1	9	M
18	Benign	B	B	B	B	M	B	B	M	M	B	7	3	B
19	Benign	B	B	B	B	M	M	B	B	B	B	8	2	B
20	Malign †	M	M	M	M	M	M	M	M	M	M	0	10	M
21	Benign	B	B	B	B	M	B	B	B	B	B	9	1	B
22	Benign	B	B	B	B	M	B	B	B	B	B	9	1	B
23 NB	Malign †	M	M	B	M	M	B	B	B	M	B	5	5	B/M
24	Malign †	M	M	M	M	M	M	M	B	M	M	1	9	M
25	Benign	B	B	B	B	M	B	B	B	B	M	8	2	B
26	Malign *	M	M	M	M	M	M	M	M	M	M	0	10	M
27	Benign	B	B	B	B	B	B	B	M	B	M	8	2	B
28	Benign	B	B	B	B	B	B	B	B	B	B	10	0	B
29	Benign	B	B	B	B	M	B	B	B	B	B	9	1	B
30	Malign †	M	M	M	M	M	M	M	M	M	M	0	10	M
31	Malign †	B	M	M	M	M	M	M	B	M	M	2	8	M
32	Benign	M	B	B	B	M	B	B	B	M	B	7	3	B
33	Malign †	M	M	M	M	M	M	M	B	M	M	1	9	M
34	Benign	B	B	B	B	B	B	B	B	B	B	10	0	B
35	Malign †	M	M	M	M	B	M	M	B	M	M	2	8	M

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THE RELIABILITY OF PATHOLOGISTS

A Study of Some Cases of Lymph Node Biopsies Showing Giant Follicular Hyperplasia or Lymphoma

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The slides from 35 cases of lymph node biopsies with the initial diagnosis of Giant Follicular Lymphoma or Giant Follicular Hyperplasia were re-examined according to selected histopathological criteria. The same slides were also sent for diagnosis to eight different trained pathologists without any information on previous diagnosis or clinical picture. The 35 patients were clinically followed up and their fates registered. There were some (2 of 19 cases) false positive diagnoses, and some (2 of 16 cases) false negative diagnoses. Differences of opinion between the pathologists were registered.

It is well known that the histopathological diagnosis of Giant Follicular Lymphoma is often difficult, and the differentiation from Reactive Follicular Hyperplasia uncertain. In the present paper an analysis will be made of a series of cases of enlarged lymph nodes submitted for biopsy diagnosis. After routine examination a diagnosis of either Giant Follicular Hyperplasia or Giant Follicular Lymphoma was given. Often a comment was added, indicating that malignancy could not with certainty be excluded. These cases are used for an analysis of the reliability of the routine diagnostic work of the pathologist.

MATERIALS AND METHODS

The material is a part of a total of about 5000 lymph nodes received at our laboratory in the years 1950 to 1959 inclusive. Out of these, 38 cases were found presenting the criteria mentioned. The 38 cases comprise 18 males and 20 females.

Clinical Development

The cases were re-examined in 1967, the course of their disease was noted and all relevant clinical, radiological and histopathological data assessed. The patients had thus an observation period of 8–13 years. It was impossible to trace three of the patients. The re-examination therefore comprised 35 patients, in which satisfactory information was available. The Norwegian Cancer Registry was as usual very helpful. We are also indebted to all the clinicians who generously gave us their help.

We classified the clinical development of our patients into two groups. Group 1 comprises 17 patients who were alive and without recurrence 8 years or more after the diagnosis and two patients who died of intercurrent disease without having any symptoms or signs of malignant lymphoid disease at the time of death. These 19 patients were classified as showing a *Benign clinical development* (see Tables 1 and 2). It is realized that 8–13 years in some cases may be insufficient for a final diagnosis of a possible lymphoma, but we decided to use this criterion. Group 2 comprises 14 patients who died of malignant lymphoid disease and two patients still alive but with signs of recurrence. In these two cases both subsequent histology and the clinical picture were undoubtedly that of a malignant lymphoid tumour. Both are alive and relatively healthy, but they have signs of malignant

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TABLE 2 *Comparison of Diagnoses and Clinical Development*

Diagnostic procedure	Clinical Development			
	Benign		*Malignant*	
	Histological classification		Histological classification	
	Benign	Malignant	Benign	Malignant
Primary diagnosis	14	5	2	14
Revised Diagnosis	18	1	1	15
Blind Test Diagnosis[b]				
Pathologist Sen	16	3	1	15
Pathologist Jun	13	6	3	13
Pathologist A	17	2	3	13
Pathologist B	17	2	1	15
Pathologist C	7	12	2	14
Pathologist D	14	5	2	14
Pathologist E	17	2	2	14
Pathologist F	13	6	10	6
Cumulative Diagnosis	17	2	1(2)	15(14)
No of cases	19		16	

these as Probably Benign and there was one undecided (5–5) These latter could be called *false negative diagnosis* It is seen that both the Revised Diagnosis two of the patho logists and the Cumulative Diagnosis" gave the same good result namely one false ne gative

DISCUSSION

It is commonly thought by informed laymen and also among many cancer research workers without medical background that the histopathological diagnosis of malignancy is easy It is true that in some tumour types almost complete agreement can be reached but that this is not always so is familiar to all pathologists As regards lymph node biopsies the diagnostic work is especially difficult, as recently clearly demonstrated by *Symmers* 1968 a and b) When *Everson & Cole* 1966 wrote their book on Spontaneous Re gression of Cancer they excluded all lym phomas from consideration

The problem of Giant Follicular Hyper plasia of infectious or reactive etiology ver sus Brill Symmers disease interpreted as the beginning of a malignant condition is a good example of this Our study reveals that in many cases uncertainty is present and dis- agreement among pathologists exists

In a recent clinical and pathological review of 64 such cases *Firat et al* (1965) came to the same conclusion 'the differences of opinion exhibited by two competent patho- logists upon review of the slides in this series of patients was striking One of them la belled too many as malignant—the other not enough Although not presented in this paper, considerable variation occurred in the check sheaths from day to day when the same pathologist reviewed the same slides

In such a situation it is important that the histopathological criteria are as strict and well defined as possible Our study has reve aled that fairly good criteria exist The good agreement between the Revised Diagnosis and the clinical course of the patients de monstrates the value of the criteria put down by *Rappaport* (1963) and *Lennert* (1964) But our study also shows that pathologists are not always able to assess and use these criteria properly It must be accepted that the daily diagnostic work in histopathology

lymphoid disease These 16 patients are classified as showing *Malignant clinical development*

Histopathological Diagnosis

Our aim was to investigate the reliability of our routine diagnostic work We therefore used the original routine sections for our studies, and some of the material was not of desired quality This was often due to the fact that the surgeons provided only small and fragmented pieces of lymph nodes, or that the fixation procedure was unsatisfactory Some of the sections accepted for the routine diagnostic work were also rather thick

The histopathological diagnosis was analyzed at three levels

1 The primary Diagnosis (*Prim*) is the designation for the original diagnosis, with the accompanying remark The main bulk of our biopsy specimens are submitted from surgeons and provincial hospitals, and often with very little clinical information The diagnoses were given by six different pathologists during a period of ten years The diagnostic criteria, the descriptions and the terminology are accordingly not uniform, but have changed from pathologist to pathologist, and with time The 35 cases can, corresponding to the terms and descriptions used, be separated into two "classes' Class B (*Benign*) (see Table 1) means that no suspicion of malignancy was expressed Class M (*Malignant*) means that malignancy was mentioned in the diagnosis as a fact or a possibility We have used this grading of the primary diagnosis because the designation "Brill-Symmers disease' or "Giant Follicular Lymphoma' was not in common use at the beginning of the period

2 This material was made the subject of a *Revised Diagnosis* (*Rev*) according to uniform criteria and without any knowledge of the clinical information or the primary diagnosis During the systematic revised diagnostic work we used the criteria as described by *Rappaport* (1963) and by *Lennert* (1964)

3 Finally the material was used again for a *Blind Test Diagnosis'* by eight different pathologists Both authors of this paper participated in this 'Blind-Test-Diagnosis , which was performed about one year after the Revised Diagnosis During the third 'Blind-Test-Diagnosis the material was again referred to two classes, namely as probably Reactive Follicular Hyperplasia (*B*), or as probably Giant Follicular Lymphoma (*M*) With this method it was possible to see the variation in the use of the diagnostic criteria between eight different pathologists and to test the reliability from one year to another of the diagnostic work of the senior and the junior author

RESULTS

The Correlation between Diagnosis and Clinical Course

Table 1 shows the clinical development and the results of the diagnostic procedures for the 35 cases studied In the second column the clinical development is characterized as malignant or benign with a † after those cases that died from malignant lymphoid disease, and an asterisk after the two cases still alive, but with malignant lymphoid disease In the next columns are shown the results of the different diagnostic procedures Then the table shows the "Final Vote' for Probably *B*enign or Probably *M*alignant histopathological diagnosis, first with the number of diagnoses for each decision, and then with a "*Cumulative Diagnosis*" based on the result of the "Vote". A detailed study of this table gives a good impression of the differences of opinion that exist between different pathologists studying the same slides There are four cases labelled "NB", these are the only cases where the "Cumulative Diagnosis differed from the clinical development

Some summarized results from Table 1 are condensed in Table 2 It is seen that 19 patients had a benign clinical course, and of these 7 to 18 cases were correctly classified as Probably Benign by the different histopathological procedures From 1 to 12 cases were classified as Probably Malignant These latter could be called *false positive diagnoses* It is seen that the lowest number of false positive diagnoses is achieved by the "Revised Diagnosis" But the "Cumulative Diagnosis and three of the pathologists come very close to this good result

16 patients showed a malignant clinical development From 6 to 15 of these were correctly classified as Probably Malignant by the different histopathological procedures 14 were classified as Probably Malignant by the "Cumulative Diagnosis", and there was one undecided (5–5) From 1 to 10 cases were wrongly classified as Probably Benign by the histopathological diagnosis The "Cumulative Diagnosis' incorrectly classified one of

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DIPHASIC INCREASE OF VASCULAR PERMEABILITY IN TURPENTINE-INDUCED INFLAMMATION IN SKIN AND MUSCULATURE OF MICE

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Inflammation was induced in the abdominal skin and musculature of mice by subcutaneous injection of turpentine The leakage of serum proteins estimated by the trypan blue technique was found to be diphasic, both in the skin and musculature The early phase was completed in about 2 hours The late phase started at about 3 hours and reached a maximum 12 to 16 hours after turpentine injection However, even at 48 hours considerable dye leakage was present By means of the colloidal carbon technique, turpentine was found to induce a diphasic labelling of venules in the skin and musculature, the early phase being slight and short lived the second marked and prolonged Labelling of capillaries occurred in the panniculus carnosus in the first half hour of inflammation At 36 and 48 hours capillary labelling occurred in a ringshaped area of the skin In the musculature capillary labelling occurred in the first 3 hours of inflammation Tissue necrosis was present in the panniculus carnosus at 14 hours in the dermis at 24 hours and in the epidermis at 48 hours of inflammation Necrosis was also present in a thin layer of the musculature close to the abscess wall after 14 hours of inflammation Inflammatory oedema developed rapidly during the second hour of inflammation and slowly during the following 22 hours Some relations between protein leakage, vascular carbon labelling tissue necrosis and oedema formation are discussed

A diphasic increase of vascular permeability has been found in inflammation induced by thermal injury (*Sevitt* 1958 *Wilhelm & Mason* 1958 1960 *Allison & Lancaster* 1959, *Wells & Miles* 1963) bacterial infection *Burke & Miles* 1958) clostridium toxins (*Elder & Miles* 1957, *Craig & Miles* 1961), ultraviolet injury (*Logan & Wilhelm* 1963) and numerous chemical irritants (*Steele & Wilhelm* 1967 *Hurley et al* 1967)

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Turpentine has been used widely to induce experimental inflammation In our laboratory turpentine-induced inflammation has been used as a model for studies of the inflammatory process in mice Turpentine injected subcutaneously into the abdominal wall of mice causes inflammation in the abdominal skin and musculature and the development within 24 hours of a well-defined abscess (*Svanes* 1964) It was considered worth while to study the changes in vascular permeability associated with this abscess formation Preliminary experiments suggested that turpentine causes a diphasic increase of vas

is not only based on the objective use of strict criteria, but there is a certain amount of "intuition", personal judgement and therapeutic philosophy involved The "intuition' is probably a quick, partly unconcious assessment of the total histological picture based *on experience and training* Our study has shown that the pathologist's "intuition" is not always correct The therapeutic philosophy will bias the optimistic mind towards malignancy in cases of doubt He will think that there is a possibility of cure and the earlier in the disease process the better The pathologist who believes that all treatment for these diseases are palliative and dangerous, will be biased towards a benign diagnosis Our study *gives an illustrating example* of this, which can be seen by comparing Pathologist C (26 malignant diagnoses) with Pathologist F (12 malignant diagnoses (see Table 2)

The conclusion may be drawn that in a diagnostic system like that used in our institute, the most accurate diagnostic and prognostic results on these types of lymph node biopsies can be achieved either by adhering *to strict* histological criteria without access to clinical information, or by showing the slide to a group of experienced pathologists and then relying upon a simple majority vote within such a group But even with these precautions, there will be some false positive and some false negative results

It is also important to add that the division into Groups and Classes made in this study may give an impression of a more definite decision than sometimes applied in daily diagnostic work Very often the pathologist does not, in cases like this, give a definite diagnosis, but writes a comment and gives *the clinicians some suggestions for further* steps to secure the diagnosis It is of utmost importance then that the channels of information between pathologist and clinicians are used and that the two groups "understand the language" they use

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DIPHASIC INCREASE OF VASCULAR PERMEABILITY IN TURPENTINE-INDUCED INFLAMMATION IN SKIN AND MUSCULATURE OF MICE

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Inflammation was induced in the abdominal skin and musculature of mice by subcutaneous injection of turpentine. The leakage of serum proteins estimated by the trypan blue technique was found to be diphasic both in the skin and musculature. The early phase was completed in about 2 hours. The late phase started at about 3 hours and reached a maximum 12 to 16 hours after turpentine injection. However even at 48 hours considerable dye leakage was present. By means of the colloidal carbon technique turpentine was found to induce a diphasic labelling of venules in the skin and musculature, the early phase being slight and short lived, the second marked and prolonged. Labelling of capillaries occurred in the panniculus carnosus in the first half hour of inflammation. At 36 and 48 hours capillary labelling occurred in a ringshaped area of the skin. In the musculature capillary labelling occurred in the first 3 hours of inflammation. Tissue necrosis was present in the panniculus carnosus at 14 hours, in the dermis at 24 hours and in the epidermis at 48 hours of inflammation. Necrosis was also present in a thin layer of the musculature close to the abscess wall after 14 hours of inflammation. Inflammatory oedema developed rapidly during the second hour of inflammation and slowly during the following 22 hours. Some relations between protein leakage, vascular carbon labelling, tissue necrosis and oedema formation are discussed.

A diphasic increase of vascular permeability has been found in inflammation induced by thermal injury (*Sevitt* 1958, *Wilhelm & Mason* 1958, 1960, *Allison & Lancaster* 1959, *Wells & Miles* 1963), bacterial infection (*Burke & Miles* 1958), clostridium toxins (*Elder & Miles* 1957, *Craig & Miles* 1961), ultraviolet injury (*Logan & Wilhelm* 1963) and numerous chemical irritants (*Steele & Wilhelm* 1967, *Hurley et al* 1967).

Turpentine has been used widely to induce experimental inflammation. In our laboratory turpentine induced inflammation has been used as a model for studies of the inflammatory process in mice. Turpentine injected subcutaneously into the abdominal wall of mice causes inflammation in the abdominal skin and musculature and the development within 24 hours of a well defined abscess (*Svanes* 1964). It was considered worth while to study the changes in vascular permeability associated with this abscess formation. Preliminary experiments suggested that turpentine causes a diphasic increase of vas

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cular permeability in both skin and musculature However, the permeability changes in the skin seemed to differ considerably from those in the musculature

The present investigation was therefore designed to study the turpentine induced changes of vascular permeability in the abdominal skin and musculature of mice over a prolonged period of time, and in particular, the cause of the different reaction in skin and musculature

MATERIAL AND METHODS

Animals Male albino mice, 4–10 months old, were used (*Hartzeit* 1961) They were fed on pellets and water ad libitum

Turpentine inflammation was produced by injection of 0 01 ml turpentine subcutaneously into the abdominal wall of the mice after shaving with a machine clipper

Dye leakage The local leakage of serum proteins caused by turpentine was estimated by the trypan blue technique 3 ml/kg body weight of a 0 5 per cent solution of trypan blue was given intravenously at different intervals after the injection of turpentine The mice were killed 20 minutes later Standard samples including all layers of the abdominal wall were removed with a cork borer with a diameter of 9 mm The skin was separated from the musculature and the content of trypan blue in the samples was determined by extraction and measurement in a spectrophotometer (*Judah* & *Willoughby* 1962 *Stanes* 1965) In this paper "dye leakage is defined as the difference between the amount of dye extracted from samples of turpentine treated mice and that extracted from samples of saline treated control mice

Vascular labelling Colloidal carbon injected intravenously causes vascular labelling of blood vessels with increased permeability In the present study the method described by *Majno et al* (1961) and *Hurley* & *Spector* (1965) was used A colloidal suspension of carbon black supplied by Gunther Wagner Pelican Werke, Hannover Germany (batch C11/1431a) to which was added 4 5 per cent gelatin and 1 3 per cent Phenol was injected intravenously in a dose of 0 1 ml/100g body weight, 20 minutes before the mice were killed The abdominal wall with the inflamed area was excised The skin was separated from the musculature placed on a piece of paper and fixed in 4 per cent neutral formaline for a minimum of 12 hours The preparations were dehydrated and cleared as described by *Hurley* & *Spector* (1965)

The vascular labelling in the cleared preparations was studied microscopically and by means of photos taken at low magnification (× 5 5) The degree of venular labelling was estimated by means of the photos The area showing venular labelling was measured and the average blackening of venules in this area was estimated by means of an arbitrary scale Venular labelling was expressed then as the product of the size and colour intensity of this area Capillary labelling was estimated in a similar way However, since the capillaries could not be clearly seen on the photos, the intensity and extent of capillary labelling had to be evaluated by microscopic examination which makes the estimation of capillary labelling somewhat less accurate than that of the venular labelling The terminology suggested by *Zweifach* (1961) for capillary vessels was used

Histology The abdominal wall with the inflamed area was cut out, placed on a piece of paper to avoid wrinkling and fixed in 4 per cent neutral formaline for a minimum of 24 hours Sections were made through the central part of the inflamed area The slides were stained with hematoxylin and eosin

Oedema Standard samples including all layers of the abdominal wall were removed as described above The samples were weighed just after removal and after drying in a thermostat at 100° C for 6 days The amount of oedema fluid in samples from turpentine treated mice was estimated as the difference between the weight of the wet sample and a calculated value representing the wet weight of a control sample with the same dry weight

$$\text{Oedema} = \text{Weight (mg) of wet sample} - \text{Weight (mg) of dried sample} \left[1 + \frac{\text{mg fluid}}{\text{mg dried tissue}} \text{ in control samples} \right]$$

RESULTS

Dye leakage Eighty eight mice were given a subcutaneous injection of 0 01 ml turpentine and 22 control mice were given a corresponding injection of saline At different intervals after the subcutaneous injection an intravenous injection of trypan blue was given to groups of 5 mice 4 turpentine treated and one saline-treated and the dye leakage was determined as described above

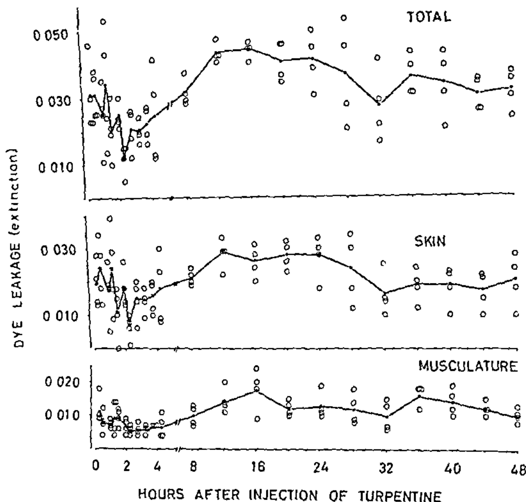


Fig 1 Leakage of circulating trypan blue into the abominal skin and musculature of mice induced by subcutaneous injection of turpentine

○ Individual values ●—● Mean values

The content of trypan blue in samples from saline treated mice showed no special trend when related to time after injection of saline, the mean extinction value, with range, being 20 (17–23) for combined samples (total) 14 (11–16) for skin samples and 6 (3–8) for muscle samples These mean values were used for calculation of dye leakage in turpentine treated mice

The results are recorded in Fig 1 The dye leakage in the skin and musculature as well as the total dye leakage (skin + musculature) is diphasic In the skin the early phase began soon after turpentine injection and was completed in about 2 hours The late phase commenced at about *3 hours*, reached a maximum 12–24 hours after turpentine injection, and then declined During the last 16 hours of the *experiment the* dye leakage remained fairly constant at a moderate level In the musculature the dye *leakage* shows a diphasic curve similar in time course to that in the skin However, in the *second phase the leakage* remained high for a longer period of time than in the skin

The correlation between dye leakage in

cular permeability in both skin and musculature However, the permeability changes in the skin seemed to differ considerably from those in the musculature

The present investigation was therefore designed to study the turpentine induced changes of vascular permeability in the abdominal skin and musculature of mice over a prolonged period of time, and in particular, the cause of the different reaction in skin and musculature

MATERIAL AND METHODS

Animals Male albino mice, 4–10 months old were used (*Hartveit* 1961) They were fed on pellets and water ad libitum

Turpentine inflammation was produced by injection of 0 01 ml turpentine subcutaneously into the abdominal wall of the mice after shaving with a machine clipper

Dye leakage The local leakage of serum proteins caused by turpentine was estimated by the trypan blue technique 3 ml/kg body weight of a 0 5 per cent solution of trypan blue was given intravenously at different intervals after the injection of turpentine The mice were killed 20 minutes later Standard samples including all layers of the abdominal wall were removed with a cork borer with a diameter of 9 mm The skin was separated from the musculature, and the content of trypan blue in the samples was determined by extraction and measurement in a spectrophotometer (*Judah & Willoughby* 1962 *Stanes* 1965) In this paper 'dye leakage is defined as the difference between the amount of dye extracted from samples of turpentine treated mice and that extracted from samples of saline treated control mice

Vascular labelling Colloidal carbon injected intravenously causes vascular labelling of blood vessels with increased permeability In the present study the method described by *Majno et al* (1961) and *Hurley & Spector* (1965) was used A colloidal suspension of carbon black supplied by Gunther Wagner Pelican Werke, Hannover Germany (batch C11/1431a) to which was added 4 5 per cent gelatin and 1 3 per cent Phenol was injected intravenously in a dose of 0 1 ml/100g body weight, 20 minutes before the mice were killed The abdominal wall with the inflamed area was excised The skin was separated from the musculature, placed on a piece of paper and fixed in 4 per cent neutral formaline for a minimum of 12 hours The preparations were dehydrated and cleared as described by *Hurley & Spector* (1965)

The vascular labelling in the cleared preparations was studied microscopically and by means of photos taken at low magnification (× 5 5) The degree of venular labelling was estimated by means of the photos The area showing venular labelling was measured and the average blackening of venules in this area was estimated by means of an arbitrary scale Venular labelling was expressed then as the product of the size and colour intensity of this area Capillary labelling was estimated in a similar way However, since the capillaries could not be clearly seen on the photos the intensity and extent of capillary labelling had to be evaluated by microscopic examination which makes the estimation of capillary labelling somewhat less accurate than that of the venular labelling The terminology suggested by *Zweifach* (1961) for capillary vessels was used

Histology The abdominal wall with the inflamed area was cut out, placed on a piece of paper to avoid wrinkling and fixed in 4 per cent neutral formaline for a minimum of 24 hours Sections were made through the central part of the inflamed area The slides were stained with hematoxylin and eosin

Oedema Standard samples including all layers of the abdominal wall were removed as described above The samples were weighed just after removal and after drying in a thermostat at 100° C for 6 days The amount of oedema fluid in samples from turpentine treated mice was estimated as the difference between the weight of the wet sample and a calculated value representing the wet weight of a control sample with the same dry weight

$$\text{Oedema} = \begin{matrix}\text{Weight (mg)}\\ \text{of wet}\\ \text{sample}\end{matrix} - \begin{matrix}\text{Weight (mg)}\\ \text{of dried}\\ \text{sample}\end{matrix} \left[1 + \begin{matrix}\text{mg fluid/mg}\\ \text{dried tissue}\\ \text{in control samples}\end{matrix} \right]$$

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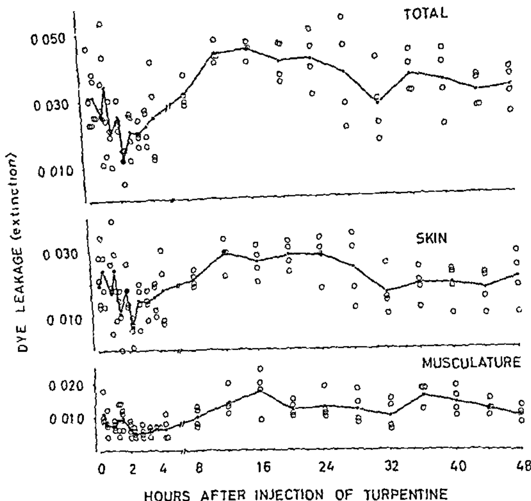


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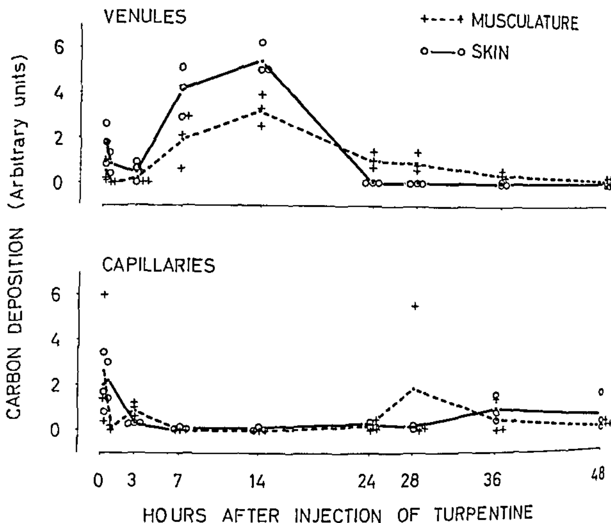


Fig Carbon labelling of venules and capillaries in abdominal skin and musculature of mice induced by subcutaneous injection of turpentine

kin and time after injection of turpentine was statistically significant during the first 2 hours (r = —0 42, P < 0 05), from two to 12 hours after turpentine injection (r = +0 75, P < 0 001) and from 20 to 32 hours after the injection (r = —0 56, P < 0 05)

The correlation between dye leakage in musculature and time after turpentine injection was statistically significant during the first 3 hours (r = —0 44, P < 0 02), from 2 to 16 hours after turpentine injection (r = +0 ... < 0 001) and from 36 to 48 hours after injection (r = —0 66, P < ...

... Eight ... Twenty seven mice ... subcutaneous injection of turpentine ... Control mice were given a ... injection of saline At different

Fig 3 Carbon labelling of venules in abdominal skin and musculature of mice induced by subcutaneous *injection* of turpentine Cleared preparations × 55

a Skin 7 hours after turpentine injection showing even labelling of venules in inflamed area

b Skin 14 hours after turpentine injection showing venular labelling in a ring shaped area

c Skin 24 hours after turpentine injection showing contour of abscess, but minimal carbon deposition

d Musculature 14 hours after turpentine injection showing intense venular labelling

e Musculature 24 hours after turpentine injection showing less extensive venular *labelling* than at 14 hours

f Musculature 48 hours after turpentine injection showing contour of abscess and a *few* labelled venules


d
e
f

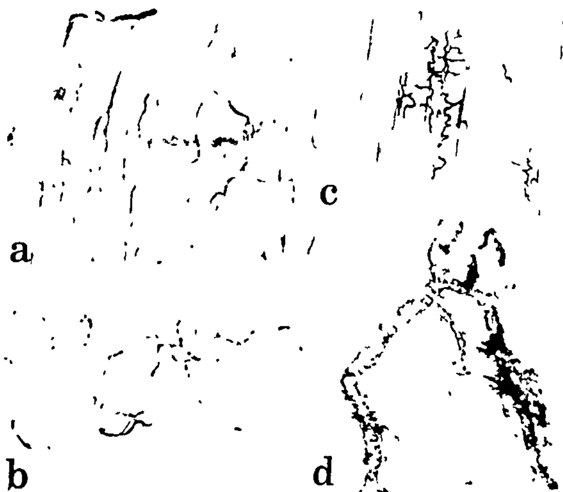


Fig 4 Carbon labelling of blood vessels in abdominal skin and musculature of mice induced by subcutaneous injection of turpentine Cleared preparations × 80

a Skin 20 minutes after turpentine injection showing faint carbon labelling of capillaries in the panniculus carnosus
b Skin 48 hours after turpentine injection showing faint labelling of capillaries in the connective tissue
c Musculature 20 minutes after turpentine injection showing heavy carbon labelling of capillaries
d Musculature 14 hours after turpentine injection showing intense venular labelling and carbon deposites in the extravascular tissue

intervals after the subcutaneous injection an intravenous injection of colloidal carbon was given to groups of 4 mice, 3 turpentine-treated, and one saline-treated, and 20 minutes later the animals were killed

The degree of vascular labelling related to time after turpenting injection is illustrated in Fig 2 In both skin and musculature the labelling of *venules* is diphasic with an early response which was slight and short-lived, followed by a second response which was more marked and prolonged

In the first 40 minutes of inflammation carbon deposition occurred in the skin in an area measuring about 70 square mm in venules less that 30μ in diameter The blackening of the vessels was moderate and the carbon was confined to the vessel walls At 3 hours the venular labelling was minimal At 7 and 14 hours of inflammation carbon deposition occurred in a 100–160 square mm area of skin in venules less than 60 μ in diameter Extravascular leakage of carbon occurred especially in venules less than 20 μ in diameter At 7 hours there was a diffuse labelling of venules in the affected area (Fig 3a), while at 14 hours the labelling was confined to a ring shaped area (Fig 3b) Mini

mal or no carbon deposition occurred in the skin in the last 24 hours of the experiment (Fig 3c)

In the first 20 minutes of inflammation carbon deposition occurred in a 20 square mm area of musculature in venules less than 20 μ in diameter The labelling was moderate and confined to the vessel walls At 40 minutes and 3 hours after turpentine injection only a minimal venular labelling was present in the musculature At 7 and 14 hours carbon labelling was found in a 60 square mm area of musculature in venules up to 70 μ in diameter (Fig 3d) At 14 hours the venular labelling was intense, with marked leakage of carbon from small venules (diameter less than 30 μ) into the extravascular tissue (Fig 4d At 24 through 48 hours after turpentine injection the venular labelling decreased gradually in the musculature (Fig 3e, f)

As *shown in* Fig 2 carbon labelling of *capillaries* occured in the skin in the first 40 minutes and the last 12 hours of inflammation In the first 40 minutes extensive capillary labelling occurred in the panniculus carnosus *(Fig 4a) At 36* and *48 hours* capillary labelling occurred in a ringshaped area of skin surrounding the abscess not involving capillaries of the panniculus carnosus Fig 4b shows the type of capillaries involved

In the musculature capillary labelling occurred in the majority of animals in the first 3 hours of inflammation while a late capillary labelling occurred only in a *couple of* animals In the musculature the labelling *of* individual capillaries was always intense the capillaries looking like a black network surrounding the muscle fibres (Fig 4c) On the *other* hand the capillaries of the skin were usually slightly or moderately blackened (Fig 4a b No leakage of carbon from capillaries was *detected*

In the *saline* treated control mice traces of *venular* labelling occurred *in a small area* of skin probably corresponding *to the injection* site in the first 40 *minutes* after the injection There was no *venular labelling* in the skin 3 36 hours after the *injection of* saline A slight venular labelling (0 4 arbitrary units) was present in the skin of the mouse killed 48 hours after the saline injection It seems unreasonable to relate this to the injection of saline, since no reaction was found 3–36 hours after the injection Venular labelling did not occur in the musculature of the control mice

Carbon labelling of capillaries was not found in the skin and musculature of saline-treated mice

Histology Fifteen mice were given a subcutaneous injection of turpentine Groups of five mice were killed 14, 24 and 48 hours after the injection

As described previously (*Stanes* 1964), turpentine injected subcutaneously into the abdominal wall of mice *induces within* 24 hours the formation of a well-defined abscess Forty-eight hours after turpentine injection the appearance *of the abscess was about the* same as that *of the 24-hour* inflammation The *superficial part of* the abscess wall is situated at *the panniculus* carnosus, the muscle fibres of which are surrounded by leukocytes *The* deeper part of the abscess wall is *situated around* the muscular fascia

At 14 hours of inflammation the muscle fibres of the panniculus carnosus were found to be *necrotic in* a restricted area *corresponding to the* abscess In the abdominal musculature *numerous* muscle fibres situated within *or close to* the abscess wall were degenerated or necrotic However, the major part of the *musculature* looked normal No evidence of *necrosis* was discovered in the dermis or epi*dermis*

At 24 hours of inflammation degeneration *and necrosis* of muscle fibres were found in *panniculus* carnosus and the musculature, *similar* in appearance and extent to that of the 14 hour inflammation In *4 of 5* animals tissue necrosis was found in a restricted area of *the dermis*, the changes being most evident in the dermal appendages *In one* mouse the epidermis was also necrotic in a circumscribed area

At 48 hours of inflammation *the* changes in the panniculus carnosus and the muscula

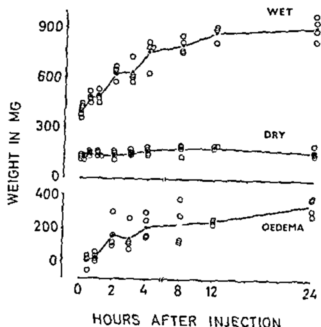


Fig 5 Weight of standard samples of tissue removed from the abdominal wall of mice given a local injection of turpentine The samples were weighed just after removal and after drying at 100° C for 6 days and the amount of oedema fluid was calculated
○ Individual values
●—● Mean values

ture were about the same as at 24 hours In 4 of 5 animals there was a circumscribed area of necrosis in both epidermis and dermis

Oedema Thirty two mice were given a subcutaneous injection of turpentine and groups of 4 mice were killed at different intervals after the injection Six control mice were not given any injection The amount of oedema in the inflamed area was estimated as described above The results are recorded in Fig 5 The weight of the wet preparations show a continuous increase during the first 24 hours after turpentine injection The weight of the dry preparations remained fairly constant during the experiment Oedema developed rapidly during the second hour of inflammation Between 3 and 24 hours after turpentine injection the oedema increased slowly

Student s t test revealed a statistically significant difference between the oedema values obtained at 50 minutes and 2 hours ($P < 0.01$) The correlation between degree of oedema and time after injection of turpentine was statistically significant from 2 to 24 hours after the injection ($r = +0.66$ $P < 0.01$)

DISCUSSION

In the present experiment turpentine was found to induce a diphasic leakage of serum proteins in the abdominal skin and musculature of mice By means of the colloidal carbon technique turpentine was found to induce a disphasic labelling of venules in the skin and musculature of mice similar in time course to that of the dye leakage Labelling of capillaries was found to be diphasic in the skin while in the musculature a late labelling occurred only in a couple of animals

In the musculature the labelled capillaries were completely black A similar capillary labelling was found by *Hurley* & *Spector* (1965) in the diaphragma of rats with turpentine induced pleurisy *Ham* & *Hurley* (1965) using a electron microscope showed that in these capillaries the carbon lay in the lumen indicating damage to endothelial cell without increase in capillary permeability In the skin of mice the carbon appeared to be bound to the walls of the capillaries as in the carbon labelling of venules It is possible therefore that the capillary labelling in the skin indicates that the permeability was increased

Consequently it is assumed that the early leakage of serum proteins in the skin induced by turpentine is due to increased permeability of venules and of the capillaries in the panniculus carnosus The maximum protein leakage 12–16 hours after turpentine injection is due to increased venular permeability while the moderate protein leakage during the last 16 hours of the experiment may be due to increased capillary permeability

In the musculature both the early and late phase of protein leakage are probably due to increased permeability of venules since the type of capillary labelling observed in the musculature cannot be expected to be associated with increased permeability

The histologic examination revealed necrosis in the panniculus carnosus at 14 hours, in the dermis at 24 hours and in the epidermis at 48 hours of inflammation At 24 hours of inflammation the carbon labelling of venules in the skin was confined to a ring shaped area It seems reasonable to explain this as a result of circulatory disturbances associated with the development of necrosis in the skin

After 24 hours of inflammation necrosis was present in the panniculus carnosus and the dermis of the skin while in the musculature necrosis was confined to a thin layer adjacent to the abscess wall, the major part of the muscle looking normal This may explain why the carbon labelling of venules in the musculature decreased gradually 24 through 48 hours after turpentine injection, while there was no venular labelling in the skin during this period

The capillary labelling confined to a ring shaped area of skin in the last 12 hours of inflammation may be the result of the tissue necrosis It seems unlikely that this should be a direct effect of turpentine on the vessels

Hurley & Spector (1965) injected turpentine into the pleural space of rats and studied the changes in vascular permeability in the diaphragma using the colloidal carbon technique They found a diphasic carbon labelling of venules which is in agreement with the present results obtained in mice However the second phase was found to reach a maximum at about 5 hours after turpentine injection while in the present experiment this maximum was reached at about 14 hours They also found labelling of capillaries which started about 45 minutes after injury reached a maximum between 2 and 5 hours and ceased by about 16 hours This differs markedly from the time course of capillary labelling induced by turpentine in the musculature of mice The difference may be due to the method of application resulting in a different concentration of turpentine at the effector cells

The leakage of serum proteins estimated by the trypan blue technique indicates that the vascular permeability is increased At a given increase in capillary permeability the leakage of proteins must be expected to depend also on other factors, such as blood flow and the hydrostatic pressure inside and outside the blood vessels The interstitial pressure in the inflamed area must be expected to increase as a result of oedema formation In the present experiment oedema developed rapidly during the second hour of turpentine inflammation Simultaneously the protein leakage decreased This observation might support the view that the falling dye leakage was due in part to increased interstitial pressure However, at 4 to 12 hours of inflammation the protein leakage increased markedly while the oedema remained fairly constant It therefore seems reasonable to conclude that under the present experimental conditions the interstitial pressure in the inflamed area does not appear to play a major role in the leakage of serum proteins

The slight changes in oedema together with the marked dye leakage 4 to 24 hours after turpentine injection suggest that the lymph drainage from the inflamed area was increased markedly, and was sufficient to remove the plasma leaking from the blood vessels

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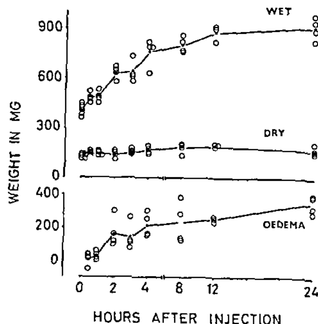


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THE METAL (SULPHIDE SILVER METHOD) AND ENZYME CYTOCHEMICAL PATTERN OF HUMAN SEMINAL SPERMATOZOA

Kerstin Boström

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Ejaculates from 200 men were examined by the sulphide silver method for the demonstration of metals and by cytochemical methods for the demonstration of lactate and succinic dehydrogenase, NADH diaphorase acid and alkaline phosphatase and nonspecific esterase By the sulphide silver method a band-shaped pattern of silver precipitates (sulphide silver pattern SSP) appeared in the middle piece of the spermatozoa 5-9 transverse or oblique bands was regarded as normal Spermatozoa classified as normally outlined as well as spermatozoa classified as abnormally outlined sometimes showed irregularities in the SSP of the middle piece The sulphide silver method thus provides a possibility for a further differentiation of spermatozoa Also by cytochemical methods for the demonstration of lactate and succinic dehydrogenase and NADH-diaphorase, a band shaped pattern appeared in the middle piece, though somewhat less distinct than the SSP A statistically significant correlation between the percentage of spermatozoa with a regular SSP of the middle piece and the percentage of motile spermatozoa was found

The light microscopic appearance of the spermatozoa is important in the evaluation of male fertility In the examination, use is made of different haematoxylin stains Such methods however give information mainly about the outer contours of the spermatozoa, but teach little about their internal structures Examination with an electron microscope would yield more information, but can as yet not be used in routine investigations on fertility

Another way to obtain information about the spermatozoa would be to examine the pattern of cytochemically demonstrable metals and enzymes The spermatozoa were therefore studied by the sulphide silver method for demonstrating metals (*Timm* 1958) (sulphide silver pattern (SSP)) as well as by cytochemical methods for demonstrating lactate dehydrogenase (LDH), succinic dehydrogenase (SDH), NADH-diaphorase, acid phosphatase (AcP), alkaline phosphatase (AlP) and nonspecific esterase (nsEs) These enzymes were chosen since they are known to occur in the spermatozoa and/or in seminal plasma (LDH, SDH, AlP and AcP *Mann* 1964, nsEs *Beckman* & *Kjessler* 1968) NADH-diaphorase was chosen since the histochemical demonstration of NAD dependent dehydrogenases is accomplished via the diaphorases (*Barka* & *Anderson* 1965)

MATERIAL

The material consisted of ejaculates from 200 men, aged 22 to 45 years subjected to investigation because of infertility In 22 cases semen was ob-

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ate in buffered acetone (Kreutz & Barka [illegible] 196?). Naphthol AS-TR phosphate (acid) (Sigma) was used as substrate and pararosaniline-HCl (Sigma) for AcP and Fast Red Violet LB (Sigma) for AlP as diazonium salt. Tris-maleate buffer 0.2 M, pH 5.0 and 9.2 respectively was used. Incubation time was 30 minutes for AcP and 60 minutes for AlP at room temperature.

Gomori's method ([illegible]). Semen was prepared, smeared, quickly air-dried, fixed in buffered formol-calcium for 15 minutes at +4° C and incubated with sodium-β-glycerophosphate. This as substrate, 0.2 M tris-maleate buffer pH [illegible] and 9.0 respectively was used. Incubation time was 10 minutes at +37° C for AcP and 60 minutes at room temperature for AlP.

Nonspecific esterase. Smears were air-dried for a few minutes, fixed in buffered formol-calcium for 15 minutes at +4° C and incubated for 60 minutes at room temperature according to *Barka* (1957b) with naphthol AS-acetate (Sigma) as substrate and Fast Red Violet LB as diazonium salt. Tris-HCl buffer 0.2 M pH 7.4 was used.

Control of reliability of the enzyme methods was performed by omission of the substrate from the incubation fluid (LDH, SDH, NADH-diaphorase) and by addition to the incubation fluid of inhibitors: sodium fluoride for nsEs and potassium arsenate for AcP and AlP (*Pearse* 1960, *Barka & Anderson* 1963).

The above mentioned methods were moreover used on washed spermatozoa in 10 cases. The spermatozoa were washed twice in physiological saline and centrifuged for 10 minutes each time at 1300 g. The spermatozoa thus washed were suspended in physiological saline to the same volume as the original volume of semen.

RESULTS

Sulphide Silver Method

Males Investigated because of Infertility

Seminal plasma. The seminal plasma in the smears was pale yellow and contained diffusely scattered silver granules.

Spermatozoa. The head was faintly yellow. Granules of silver were found both in the apical part and basally around vacuoles as well as in the absence of vacuoles (Fig. 1 a–c). Silver granules were demonstrated in heads of normal and abnormal configuration. No relation was found between the occurrence of silver granules and any particular type of head abnormality. Silver granules were found more often in the head when granules were present in the surrounding plasma.



Fig. 1. Cytochemical examination of human seminal spermatozoa. Metal sulphide silver method. a) pattern. A regular band-shaped pattern of silver precipitates in the middle piece. Sparse silver deposits in the head. × 4700. b) Irregular pattern of bands and granules in the middle piece. Granular silver deposits in the head. × 4700. c) Irregularly arranged silver precipitates in the middle piece. Dense silver deposits in the apical part of the head. × 4700. d–f) Head stained with haematoxylin. Regular (d) and irregular (e, f) pattern of silver precipitates in the middle piece. × 4700.

Scattered silver granules were found in the tail.

The SSP of the middle piece differed from that in the head and tail. Under lower magnification the middle piece was distinctly black whether or not the seminal plasma contained silver deposits. Under higher magnification the silver deposits were found to be arranged in transverse or oblique bands (Fig. 1 a–f). The number of bands as well as their arrangement varied. Sometimes bands coalesced or granules were found instead of

tained on 2 different occasions In addition eja culates from 10 fertile men were examined

METHODS

Routine Morphological Examination

The production of ejaculates and the morphological examination was performed as described in a previous publication (*Bostrom & Andersson* 1971) The appearance of the spermatozoa was judged with the aid of the given scheme with the following additions a middle piece was regarded to be of abnormal appearance if it was duplicate, short, *i e* less than 5 μ long, or long *i e* more than 9 μ long Spermatozoa with excess of cytoplasm were grouped separately

Cytochemical Examination

Semen was examined within 4 hours of ejaculation

Sulphide silver method In order to find out the optimal conditions for a positive result in the examination of spermatozoa, before the so called physical development, seminal smears were treated with hydrogen sulphide solutions with a pH range from 1 to 10 The largest quantity of precipitated silver was obtained by a yellow ammonium sulphide solution A freshly prepared colourless ammonium sulphide solution did not give a satisfactory result (cf *Brunk et al* 1968) The following method proved suitable

Semen was smeared on a cover slip which without airdrying was stored for 30 minutes at room temperature in a 70 per cent ethanol solution containing the above mentioned ammonium sulphide solution in a final concentration of 2 per cent (pH 8 0 8 5) The smears were afterwards thoroughly rinsed in 96 per cent ethanol and in glass distilled water, placed side by side in Petri dishes and covered immediately with the developer The Petri dishes were covered with a lid to prevent evaporation and placed in the dark at room temperature for 2½ to 3½ hours The composition of the developer (*Voigt* 1959) was

A 0 2 ml 10 per cent silver nitrate solution (water solution) + 20 ml gum arabic solution (1 part by weight of gum arabic (in pieces) + 5 parts by weight of glass distilled water Some thymol crystals were added to prevent growth of bacteria The solution was stored in the refrigerator for at least 14 days before use)

B Citric acid 1 g + hydroquinone 0 4 g + 20 ml glass distilled water (freshly prepared solution)

Immediately before use 2 ml of solution B was added to solution A The mixture was shaken thoroughly for about half a minute

After the development the smears were rinsed in running tap water and in glass distilled water dehydrated and mounted in Canada balsam dissolved in xylol (DPX® is unsuitable because black silver precipitates soon become pale, probably because of oxidation)

Smears not treated with ammonium sulphide but fixed in ethanol and developed, showed only single scattered silver precipitates around the spermatozoa

No attempts were made to analyse the chemical composition of the substances demonstrated

Enzymes

The methods, described by the authors cited below, were tried in order to find the procedure which in our hands would give the best results The fixation, composition of the incubation fluid and the incubation time were varied The following methods gave the best results

Succinic dehydrogenase Semen was frozen in a mixture of propane and propylene (Gasol®, ESSO) cooled by liquid nitrogen thawed and smeared on an object glass on a surface measuring 2 5 × 2 5 cm The smears were rapidly air dried and incubated in accordance with the method described by *Nachlas et al* (1957)

Sodium succinate (Sigma)	1 0 M	0 1 ml
Tris HCl buffer	0 5 M pH 7 4	0 65 ml
Nitro Bt (Sigma) solution	0 4 %	0 25 ml

To each smear was added 0 3 ml of the incubation fluid The smears were incubated for 60 minutes at +37° C, fixed in 10 per cent saline formalin for 30 minutes and mounted in 50 per cent polyvinyl pyrrolidone (PVP K 30) (Fluka) solution (*Burstone 1957 a*)

Lactate dehydrogenase Smears were prepared and incubated according to *Nachlas et al* (1958)

Sodium lactate (Sigma)	1 0 M	0 1 ml
Magnesium chloride	0 05 M	0 1 ml
NAD (Sigma) solution	0 4 %	0 1 ml
Sodium cyanide	0 1 M	0 1 ml
Tris HCl buffer	0 2 M pH 7 4	0 35 ml

2 g PVP K 15 (Fluka) was added (*Altman & Chayen* 1965) pH was adjusted to 7 4 and 0 25 ml Nitro Bt solution was added Incubation time was 30 minutes at +37° C

NADH diaphorase Smears were prepared and incubated according to the method described by *Scarpelli et al* (1956)

NADH (Sigma) solution	1 %	0 1 ml
Tris HCl buffer	0 2 M pH 7 4	0 65 ml

PVP was added pH adjusted and Nitro Bt solution added as for LDH The smears were incubated for 30 minutes at +37° C

Acid and Alkaline Phosphatase

Azo dye method (*Barka & Anderson* 1963 *Burstone* 1958) Semen was frozen thawed smeared and fixed for ½ minute at room tempera

SSP of the middle piece and living and dead spermatozoa No significant correlation was found

Washed spermatozoa The SSP of the middle piece was unchanged while the silver precipitates outside the spermatozoa were more scanty

Repeated seminal analyses The standard deviation of spermatozoa with a regular SSP on the two occasions was 5 per cent The standard deviation of the percentage of motile spermatozoa was 11 per cent

Fertile Men

The SSP in the middle piece was regular in on the average, 54 per cent of all normally outlined spermatozoa with a range in the single sample of 39 to 70 per cent and in on the average, 30 per cent of all abnormally outlined spermatozoa (range 13 to 50 per cent)

No significant correlation was found between the percentage of motile spermatozoa and the percentage of spermatozoa with a regular SSP of the middle piece, neither between the occurrence of living and dead spermatozoa and the SSP of the middle piece

Lactate Dehydrogenase

Seminal plasma In the smears the seminal plasma appeared pale blue and granules of formazan were demonstrated but were less dense than silver granules demonstrated by the sulphide silver method

Spermatozoa Deposits of formazan were found in the apical part of the head and basally (Fig 2 a–c), in heads of normal as well as abnormal configuration No relation was found between the occurrence of formazan and any particular type of head abnormality A few irregularly scattered formazan granules were seen in the tail

The deposits of formazan were always most dense in the middle piece which, under lower magnification, appeared blue Under higher magnification a pattern of transverse or oblique bands was discernible, somewhat less

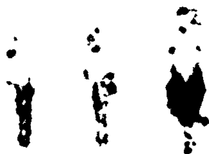


Fig 2 Lactate dehydrogenase pattern a) Band shaped pattern of formazan deposits in the middle piece Granules of formazan in the head × 4700 b) Irregular pattern of bands and granules in the middle piece × 4700 c) Excess of cytoplasm with dense deposits of formazan Granules of formazan in the head × 4700

distinct than the SSP (Fig 2 a–c) Mostly, the middle piece showed bands as well as granules or was homogenously blue When the substrate was omitted from the incubation fluid, no deposits of formazan were found in the spermatozoa

An attempt was made to classify the spermatozoa as regular and irregular according to the formazan pattern in the same way as described for the SSP A regular formazan pattern was demonstrated in, on the average, 17 per cent of all normally outlined spermatozoa, in about 9 per cent of those with an abnormally outlined head, in about 8 per cent of those with an abnormally outlined middle piece, in about 1 per cent of those with an abnormally outlined tail and in about 1 per cent of spermatozoa with excess of cytoplasm (Table 1) The cytoplasm was sometimes pale and sometimes intensely blue

No significant correlation was demonstrated between the motility of the spermatozoa and their formazan pattern of the middle piece, neither between the occurrence of living and dead spermatozoa and the formazan pattern Nor was any significant correlation found between the SSP of the middle piece and its LDH pattern

TABLE 1 *Appearance of Metal (Sulphide Silver Method) Pattern (SSP) and Lactate Dehydrogenase Pattern (LDHP) of the Middle Piece in Relation to Configuration of Spermatozoa*

	Normally outlined spermatozoa	Spermatozoa with abnormally outlined head	middle piece	tail	Spermatozoa with excess of cytoplasm
Average percentage of spermatozoa with a regular* SSP	58	33	24	26	7
Average percentage of spermatozoa with a regular LDHP	17	9	8	1	1

* Regular pattern of the middle piece denotes that silver precipitates and deposits of formazan are fairly regularly arranged in transverse or oblique bands.

bands. Sometimes bands or granules were missing and the middle piece was pale.

The spermatozoa were classified as regular or irregular according to the SSP of the middle piece. Spermatozoa whose middle piece showed transverse or oblique fairly regularly arranged bands were regarded as regular. In each smear 100 spermatozoa were counted. The reliability of this counting was tested in counts repeated 10 times on the same slide. The standard deviation proved to be 4.5.

SSP of the middle piece and configuration of spermatozoa. Normal configuration. The middle piece contained 5–9 bands. The SSP was regular in, on the average, 58 per cent of all normally outlined spermatozoa with a spread in the individual case between 35 and 75 per cent (Table 1).

Abnormally outlined head. Spermatozoa with an abnormally outlined head (middle piece and tail of normal configuration) showed a regular SSP in, on the average, 33 per cent of the entire material (range 18 to 56 per cent) (amorphous head 31 per cent, tapering head 26 per cent, small head 40 per cent, round head 30 per cent, duplicate head 32 per cent and large head 26 per cent).

Abnormally outlined middle piece. Spermatozoa with a short or long middle piece, but otherwise of normal configuration were recorded. Those with a duplicate middle piece had also a duplicate tail.

In spermatozoa with a short middle piece the number of bands varied between 1 and 4. In those with a long middle piece the number of bands was always more than 9. The largest number demonstrated was 18.

A regular SSP was found in, on the average, 24 per cent of all spermatozoa with an abnormally outlined middle piece (range 0 to 41 per cent).

Abnormally outlined tail. Spermatozoa with an abnormally outlined tail but otherwise of normal configuration were recorded.

A regular SSP was found in, on the average, 26 per cent of the entire material (range 0 to 50 per cent).

Excess of cytoplasm. Spermatozoa with excess of cytoplasm but otherwise normally outlined were recorded.

A regular SSP was found in, on the average, 7 per cent of all spermatozoa. The cytoplasm was sometimes pale and sometimes densely covered with silver deposits.

SSP of the middle piece and motility of spermatozoa. In 48 per cent of all cases with good motility, in 72 per cent of those with moderate motility and in 91 per cent of those with poor motility, less than half of the spermatozoa showed a regular SSP.

A significant correlation (correlation coefficient 0.4, $p < 0.05$) was demonstrated between the percentage of spermatozoa with a regular SSP and the percentage of motile spermatozoa.

Adhering substances from the seminal plasma may be difficult to remove from the spermatozoa despite thorough washing (*Mann* 1964)

Therefore in the present investigation it was not attempted on the basis of the cytochemical picture to draw any conclusions about the structures to which the substances demonstrated were localized The aim of the investigation was to study the spermatozoa with a view to the relation if any between their cytochemical appearance and their configuration motility and the occurrence of living and dead spermatozoa

In the present investigation washing of the spermatozoa had no effect on the SSP or formazan pattern of the middle piece But in washed spermatozoa examined by methods for the demonstration of AcP and AlP hardly any reaction products were formed This might suggest that in contrast to the substances demonstrated by reaction products obtained by the methods for AcP and AlP those demonstrated by the presence of silver precipitates or deposits of formazan were localized to the middle piece of the spermatozoa

Another important factor to be borne in mind in the interpretation of the cytochemical findings on spermatozoa is the well known fact that variations in the techniques may produce large differences in the results This may explain for example the differences in results of cytochemical examinations on human ejaculates regarding AcP With the Gomori method *Wislocki* (1950) demonstrated activity of AcP in the spermatozoal head middle piece and tail On the other hand *La ouski* (1968) found the activity of AcP to be confined to the acrosome in contact with the nuclear cap and *Mann* (1964) found that galea capitis showed only a weak AcP activity

Using the sulphide silver method silver precipitates were found in the spermatozoal head middle piece and tail The occurrence of silver precipitates in the head and tail was sporadic and no characteristic arrangement was found No relation was demonstrated between the occurrence of silver deposits in the head and its configuration In the middle piece however deposits of silver were almost constantly found and showed a characteristic arrangement Interest was therefore focused on the middle piece which showed a pattern of transverse or oblique bands of black silver

A band shaped pattern in the middle piece was also visible if the methods by which to demonstrate LDH SDH and NADH diaphorase were used Using cytochemical methods for the demonstration of oxidoreductases in human spermatozoa previous authors (*Kothare & deSou a* 1958 *Kothare et al* 1961 *Balogh & Cohen* 1964 *Hrudka* 1965) demonstrated granules of formazan in the middle piece but stated nothing about a band shaped pattern *Kothare & deSou a* (1958) found that the middle piece of abnormally outlined spermatozoa sometimes contained few or no granules of formazan In the present investigation interest was focused on the appearance of the SSP since it was more distinct and easier to evaluate than the formazan pattern and since as mentioned above human spermatozoa had not previously been examined by the sulphide silver method

It seems possible that the band shaped pattern in the middle piece is identical with the spiral structure previously discussed by many light microscopists and considered to be of mitochondrial origin

The appearance of the SSP of the middle piece varied The spermatozoa were therefore classified as regular or irregular In a normally outlined spermatozoon with a regular SSP of the middle piece 5–9 bands of fairly equal thickness were found The sulphide silver method thus offered a further possibility of differentiating spermatozoa It was remarkable that spermatozoa which according to conventional criteria were regarded as normally outlined sometimes showed irregularities in the SSP of the middle piece

These findings emphasize the problem what should be regarded as a "normal" sper

Washed spermatozoa The formazan pattern of the spermatozoa was largely the same as before washing while deposits of formazan were rarely seen in the surroundings of spermatozoa

NADH Diaphorase and Succinic Dehydrogenase

The formazan pattern of the spermatozoa and seminal plasma was largely the same as that described for LDH As regards SDH the seminal plasma and the spermatozoa were paler than that of LDH and deposits of formazan in the head were sparse

The formazan pattern of washed spermatozoa was roughly the same as that of unwashed

Nonspecific Esterase, Acid and Alkaline Phosphatase

The distribution of the reaction products in the spermatozoal head, middle piece and tail was most irregular and a pattern like that described for the previous methods did not appear Reaction products in the seminal plasma were abundant in the case of AcP but sparse in cases of AlP and nsEs

After washing reaction products in spermatozoa and their surroundings were largely missing with regard to AcP and AlP and scanty regarding nsEs

DISCUSSION

In the present investigation seminal spermatozoa were examined by the sulphide silver method for the demonstration of metals and by cytochemical methods for the demonstration of LDH SDH, NADH diaphorase AcP AlP and nsEs Interest was focused on the sulphide silver method since human spermatozoa according to our knowledge had not previously been examined by this method

The sulphide silver method is regarded as the one most sensitive for histochemical demonstration of metals (*Timm* 1958) Treatment of tissues with hydrogen sulphide precipitates metals as sulphides In a following so called physical development these metal sulphides act like ' germs (cf germs in a photographic plate (*Liesegang* 1928 *Voigt* 1959)) The resulting silver precipitates denote the localization of substances precipitable with sulphide ions It is generally known that precipitation of the various metal sulphides is optimal at a certain pH which is specific for each metal sulphide (*Stegner* & *Fischer* 1957) The tissues may however be destroyed by strongly acid or alkaline sulphide solutions Therefore, the tissues were previously treated with a hydrogen sulphide ethanol solution or with hydrogen sulphide gas without addition of acid or alkali (*Timm* 1958 *Voigt* 1959) But such conditions are not optimal for complete precipitation of all metal sulphides Precipitation of sulphides in spermatozoa visible as silver deposits after the so called physical development was largest when seminal smears were treated with a yellow ammonium sulphide ethanol solution with a pH between 8 0 and 8 5 This treatment produced no change in the configuration of the spermatozoa

In enzyme and metal cytochemical investigations the question arises which are the cellular structures to which the final reaction products are localized? For several reasons caution must be exercised before conclusions are drawn from the cytochemical findings concerning the localization of the substances demonstrated The substances may be originally bound to the cells they may become bound to the cells during the cytochemical reaction because of local affinity to certain cellular structures or the cells may be contaminated with substances from the surrounding medium Furthermore the site of the final reaction products may be different from the original site of the substance in question because the localization of the substance may change during the reaction or the substance may diffuse into the surrounding medium (*Barka* & *Anderson* 1965) All these difficulties may be encountered in the cytochemical examination of spermatozoa in semen especially since the seminal plasma is rich in enzymes and metals (*Mann* 1964)

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matozoon? Moreover, the question arises whether spermatozoal function varies with the appearance of the SSP Indeed, one might imagine that an irregular SSP of the middle piece would be contributory to infertility of the male, *in analogy with deviation* of the spermatozoal appearance The present material is not sufficient to allow any conclusions concerning the problem whether the appearance of the SSP of the middle piece may be of significance for the fertility of the male Such conclusions require a much larger material and, above all, a follow-up of fertility It is, however, tempting to assume that the SSP of the middle piece is related to the motility of the spermatozoa and the investigation has produced evidence for the existence of such a relation

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with haematoxylin-eosin, Unna's elastic stain and according to van Gieson

Histochemical Examination

Sulphide silver method 4 mm thick tissue specimens from testis efferent ducts and epididymal duct were placed in a moist chamber at room temperature and exposed to a slow stream of hydrogen sulphide for 20 minutes The specimens were frozen, kept in small air tight boxes at —20°C for 24–48 hours and cut in a cryostat into 10 μ thick sections The sections were placed on cleaned cover slips and without air-drying, transferred to a yellow ammonium sulphide ethanol solution for further treatment in the way used for ejaculates (*Boström* 1971 a)

Smears of untreated, frozen and thawed tissue from testis efferent ducts and epididymal duct, were treated in the way described for ejaculates

No attempts were made to analyse the chemical composition of the substances demonstrated

Succinic dehydrogenase (SDH) lactate dehydrogenase (LHD), NADH diaphorase, acid phosphatase (AcP) and alkaline phosphatase (AlP) 4 mm thick tissue specimens were frozen stored and cut in a cryostat In the examination for SDH LDH and NADH diaphorase, the sections were air dried for a minute before incubation Incubation fixation and mounting were done as described for ejaculates In the examination for AcP and AlP, sections were fixed in acetone for 30 minutes (15 minutes —20°C and 15 minutes at room temperature) Incubation and mounting were performed in the way described for ejaculates but the Gomori technique was not used for the tissue

Nonspecific esterase 4 mm thick tissue specimens were fixed in buffered formol-calcium for 16–24 hours at +4°C and afterwards rinsed for 24–48 hours at +4°C in 7 per cent sucrose Tris buffer pH 7 1 The specimens were cut in a cryostat incubated and mounted as described for ejaculates

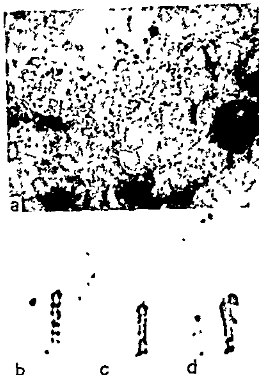


Fig 1 Metal (sulphide silver method) pattern a) Testicular tubulus Silver deposits abundant in Sertoli cells, moderate or sparse in spermiogons, spermiocytes and spermids Tubular lumen contains silver granules Spermatozoa visible with deposits of silver in the middle piece Nuclei are stained with haematoxylin × 430 b-d) Spermatozoa in efferent ducts (tissue smears) A band shaped pattern appears in the middle piece The pattern is fairly regular (b, c) or irregular (d) Head is not visible (b c) or faintly visible (d) Precipitates of silver around the spermatozoa b-d) × 4000

RESULTS

Morphological examination On gross examination the testis and epididymis were normal At the microscopic examination the testis showed mild degeneration in 24 cases and moderate in 2 (for definition, see *Boström* 1971 b) In 4 cases single foci of inflammation were found in the epididymis

Histochemical examination With the sulphide silver method and the methods for demonstrating SDH LDH NADH diaphorase, AcP and nsEs reaction products occurred in the testicular tubules and interstitial tissue, efferent and epididymal ducts Regarding AlP, reaction products were almost missing in the epithelium Interest was focused on the appearance of the epithelium and the contents of the tubules and ducts

Sulphide silver method In all testicular tubules, silver precipitates were abundant in Sertoli cells and moderate or sparse in spermiogons, spermiocytes and spermids (Fig 1 a) The granules were diffusely distributed in the cytoplasm In spermids maturing to spermatozoa the granules were scattered in the cytoplasm Silver precipitates appeared

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THE METAL (SULPHIDE SILVER METHOD) AND ENZYME CYTOCHEMICAL PATTERN OF HUMAN SPERMATOZOA IN TESTIS AND EPIDIDYMIS

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Human testis and epididymis obtained at autopsy after sudden death were studied by the sulphide silver method for the demonstration of metals and by histochemical methods for the demonstration of succinic and lactate dehydrogenase NADH diaphorase acid and alkaline phosphatase and nonspecific esterase By the sulphide silver method and methods for demonstrating succinic and lactate dehydrogenase and NADH diaphorase a band shaped pattern appeared in the middle piece of spermatozoa from testis and epididymis of the same appearance as that previously found in spermatozoa from ejaculates Irregularities of the band shaped pattern of the middle piece were found already in the testis

In a previous investigation (*Boström* 1971 a) on human ejaculates using the sulphide silver method, band shaped precipitates of silver appeared in the middle piece of the spermatozoa Also when cytochemical methods for the demonstration of succinic and lactate dehydrogenase and NADH diaphorase were used, a band shaped pattern was discernible in the middle piece The appearance of the pattern varied

The question arose whether the substances demonstrated in the middle piece were originally bound to the spermatozoa in the testis or whether they became bound to the spermatozoa later, either during the passage of the spermatozoa through the genital tract or in the semen Another question was when do the previously described variations in the pattern appear?

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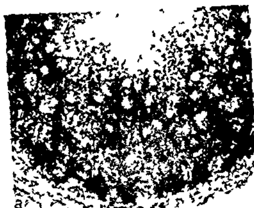
Therefore, the human testis and epididymis were examined by the same methods as the ejaculates (*Boström* 1971 a)

MATERIAL

Necropsy specimens were obtained within 3 hours of death from 26 males aged 16 82 years (one 16 years old one 26 years two 30 39 years one 43 years seven 50 59 years six 60 69 years six 70 79 years and two 80 89 years) Eighteen had died natural deaths (cardio cerebro vascular diseases in 17 bronchial asthma in 1) and 8 unnatural deaths (*7 traffic accidents 1 intoxicated with barbituric acid*) In examination of necropsy specimens obtained within a few hours of death the histochemical picture was as distinct as in examination of specimens obtained at operation within a few minutes of surgical removal

METHODS

Routine morphological examination Tissue specimens were fixed in Bouin s fixative and stained



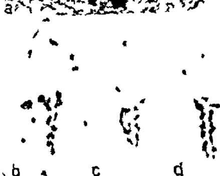


Fig. 2 Lactate dehydrogenase pattern a) Testicular tubulus. Abundant deposits of formazan in the epithelium. No spermatozoa visible × 430. b-d) Spermatozoa in efferent ducts (tissue smears). A pattern of bands and granules in the middle piece. Head contains scattered deposits of formazan. Deposits of formazan around the spermatozoa. b-d) × 4000

lumnar and cuboidal epithelium of the efferent ducts and deposits of azo dye were abundant in the columnar epithelium of the epididymal duct. Granules of azo dye were found in the lumen.

Spermatozoa of the testis and epididymis contained scattered diffusely distributed granules of azo dye in the same arrangement as that in ejaculates.

Nonspecific esterase In the testicular tubules scattered granules of azo dye were found in the epithelium, in residual bodies of cytoplasm and in the lumen. The pattern of the epididymal epithelium was similar to the formazan picture.

Spermatozoa in the testis and epididymis contained irregularly distributed granules of azo dye in the same arrangement as that in ejaculates.

DISCUSSION

Cytochemical methods used for the demonstration of enzymes and metals on human ejaculates revealed reaction products in the spermatozoa (*Duijn* 1954, for further references see *Bostrom* 1971 a). With the sulphide silver method for the demonstration of metals and methods for demonstrating SDH, LDH and NADH diphorase the substances demonstrated in the middle piece were found to be arranged in a band shaped pattern of varying appearance (*Bostrom* 1971 a).

The question arose first whether the substances were demonstrable in the spermatozoal middle piece already in the testis, second whether a band shaped pattern appeared already in the testis and third whether variations in the possible pattern were found already in the testis.

In previous investigations on the adult human testis and epididymis regarding the above mentioned enzymes interest was focused mainly on the epithelium and the interstitial tissue, little or no attention being given to the spermatozoa (*Montagna* 1952, *Jirasek* & *Raboch* 1963, *Tice* 1963, *Mancini et al* 1965, *Sharma* & *Bharadwaj* 1966, *Grino* & *Reissenweber* 1967, *Koudstaal et al* 1967 a, b, *Sengoku* 1967, *Kohl* 1968, *Bandmann* 1969).

With the isotope technique *Wetterdahl* (1958) found that zinc is incorporated in the rat spermatozoa already in the testis and this probably holds good also for human beings (*Mann* 1964). By the sulphide silver method *Timm* & *Schulz* (1966) demonstrated metals in the rat spermatozoa.

In the present investigation substances previously demonstrated in the middle piece of spermatozoa in ejaculates (*Bostrom* 1971 a) were found in the middle piece of spermatozoa also in the testis. Thus the substances seem to be bound to the spermatozoa already in the testis probably during their development.

in residual bodies of cytoplasm and the lumen often contained numerous granules

The histochemical pattern of the testis was the same whether or not spermiogenesis appeared normal When the tubular epithelium was reduced it did not differ cytochemically from normal

In the epididymis silver precipitates were found both in the lumen and in the epithelium The amount of silver in the epithelial cells varied as did that in the lumen

A pattern of silver precipitates (sulphide silver pattern SSP) was found in the spermatozoa in the testis and epididymis (Fig 1 b d) The appearance of the SSP was the same in the testis and the epididymis and the same as that in ejaculates The picture was the same in tissue sections and in tissue smears Thus the head was pale or yellow and the head and tail contained irregularly distributed silver precipitates while a band shaped pattern appeared in the middle piece With the sulphide silver method substances were thus demonstrable in the spermatozoal middle piece already in the testis

The appearance of the SSP of the middle piece sometimes varied both in the testicular and epididymal spermatozoa Attempts were therefore made to classify spermatozoa in the way described for ejaculates These attempts however proved unsuccessful in testicular sections and smears because silver granules were often abundant around the spermatozoa In sections and smears from the epididymis silver precipitates around the spermatozoa were less abundant though the picture was less distinct and the evaluation more difficult than that in ejaculates

In the efferent ducts a regular SSP in the middle piece was found in on the average 53 per cent(range 40 to 63 per cent) of all normally and in, on the average 33 per cent (range 20 to 44 per cent) of all abnormally outlined spermatozoa In the epididymal duct a regular SSP of the middle piece was found in, on the average 61 per cent (range 43 to 70 per cent) of all normally and in on the average 37 per cent (range 19 to 54 per cent) of all abnormally outlined spermatozoa

As mentioned above the testes showed mild degeneration in 24 of the 26 cases and moderate in 2 No correlation was found between the distribution of the degenerative lesions in the testes on one hand and the percentage of respectively normally and abnormally outlined spermatozoa with a regular SSP in the middle piece on the other

Lactate dehydrogenase NADH diaphorase and succinic dehydrogenase The formazan pattern of these enzymes was roughly the same but the blue colour least intense in the case of SDH In the testicular tubules formazans were abundant in the epithelium and in the lumen and were diffusely distributed in the germinal cells (Fig 2 a) In spermids maturing to spermatozoa formazans occurred diffusely distributed in the cytoplasm Deposits of formazans appeared in residual bodies of cytoplasm

In the efferent ducts the formazans were often accumulated apically or basally in the columnar and cuboidal epithelium while in the epididymal duct formazans were diffusely distributed in the cells

The formazan pattern of the spermatozoa was the same in the testis and the epididymis and of the same appearance as that in ejaculates Thus formazans were diffusely scattered in the head and tail while a band shaped pattern was discernible in the middle piece (Fig 2 b d) The often abundant occurrence of formazans in the surroundings of the spermatozoa especially in the testis made evaluation in tissue sections and smears less reliable than in smears from ejaculates Irregularities in the formazan pattern of the middle piece seemed to appear to roughly the same extent in spermatozoa in the efferent ducts as in spermatozoa in the epididymal duct and at roughly the same frequency as that in ejaculates

Acid phosphatase The azo dye pattern of the tubular epithelium of the testis was similar to the SSP Spermids maturing to spermatozoa contained granules of azo dye without any characteristic localization Granules of azo dye were found in the tubular lumen

Deposits of azo dye were found in the co

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IN VITRO STUDIES OF CHICKEN LYMPHOID CELLS

3 *The Mixed Spleen Leucocyte Reaction with Special Reference to the Effect of Bursectomy*

GUNNAR V ALM

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A tissue culture technique for the study of the mixed leucocyte reaction (MLR) occurring between allogeneic chicken spleen cells was developed Some features of the *one way* MLR, using mitomycin C-treated spleen cells as stimulators and the two-way MLR were characterized Cells forming antibody to histocompatibility or other antigens common to lymphoid cells and erythrocytes could not be detected *in the* MLR Spleen cells from hypogammaglobulinaemic bursectomized irradiated chickens reacted normally both in the two-way and one way MLR It is suggested *that the* MLR occurs between thymus dependent lymphoid cells or between such cells and macrophages Bursa dependent lymphoid cells appear to play a non-essential role if any, in the MLR.

...al (11) described the mixed leuco- ...tion (MLR) that occurs between ...onar and ...ic lymphocyte populations in tis- ...t ducts and The MLR clearly is an immune ...ent in the co...se is does not occur between ...mal duct Gr...m identical twins (11) or ..., the lumen ..., of mice or rats (16) Nor ... Spermatozoa of parent F_1-hybrid combina- ...ontained scattered lymphocytes are obtained ...granules of azo dye in ly made tolerant to the ...as that in ejaculates

Nonspecific esterase ...p be based on differ- ...bules, scattered granules ...patibility antigen found in the epithelium in ...n (6), H-2 *in* of cytoplasm and in the lumen of the epididymal epithelium ...by the grants the formazan picture ...71 01 and Spermatozoa in the testis and ... Grant) contained irregularly distributed grand...

mice (17), AgB *in* rats (30) and presumably B in chickens (28, 31) It can be demonstrated with lymphoid cells from several species and anatomical sources (36) A surprisingly large percentage of the lymphoid cells are reactive in the MLR, figures *of at least* 0 05–0 5 per cent (7) and 2–4 *per cent* (37) have been given

Mitomycin C-treatment (9) or x irradiation (21) of lymphoid cells prevent their proliferation but not the ability *to* stimulate in the MLR. This fact provides the basis for the "one way" MLR.

Lymphocytes from thymectomized rats (29, 38) and from human subjects with lymphopenia agammaglobulinaemia show impaired responses in the MLR (8) This suggests that the responding lymphocytes *in the* MLR are thymusdependent Lymphocytes

Experimental plan and calculations

The mixed leucocyte cultures usually contained 10^7 spleen cells, half of them from each of two animals or all from one animal In the one way cultures half of the cells whether autochthonous or allogeneic, were mitomycin C treated

In the two-way MLR the increases of the H^3-TdR incorporation, expressed as counts per minute per culture (c p m), caused by allogeneic cells were calculated according to the formula

$$A_nB_n - \frac{A_{2n} + B_{2n}}{2}$$

where $A_n B_n$ is the c p m in the mixed culture and A_{2n} and B_{2n} c p m in the two cell populations cultured alone n denotes the number of cells per culture

In the one way MLR the stimulation caused by the allogeneic mitomycin C treated cells (c p m per culture) was calculated according to the formula

$$A_nB_{n\,m} - A_nA_{n\,m}$$

were as before A and B are the two cell populations and n denotes the number of cells per culture m indicates the mitomycin C treated cells

RESULTS

Effect of total cell concentration on the MLR Spleen cells from strain 161 and 131 chicks were cultured at different total cell concentrations In the one way MLR only the reaction of strain 161 cells to mitomycin C-treated strain 131 cells was examined The cell cultures were all harvested on day 5

Fig 1 shows the results from one representative experiment with the two way MLR and one with the one-way MLR In both, the H^3 TdR incorporation increased in an approximately linear fashion with increasing cell concentrations

Effect of the culture time on the MLR Two-way and one-way mixed cultures containing each a total of 10×10^6 spleen cells, half from chick strain 161 and half from strain 131, were initiated and harvested 3, 5 and 7 days later Fig 2 demonstrates the results The increase of the H^3-TdR incorporation caused by allogeneic cells increased between day 3 and 7 in an approximately linear fashion

Cultures containing a total of 10×10^6 spleen cells and harvested on day 5 were therefore chosen for further experiments

Evaluation of the efficiency of the mitomycin C treatment *Bach & Voynow* (9) demonstrated that mitomycin C effectively prevented the DNA synthesis in cultured human peripheral lymphocytes Some data to the opposite have been reported (19, 35)

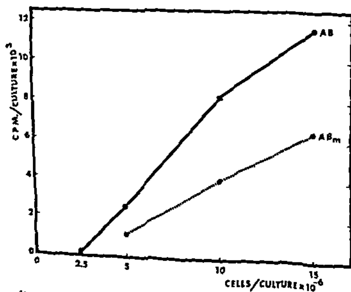


Fig 1 The effect of the total number of spleen cells per culture on the increase of the H^3 TdR incorporation (c p m per culture) in the two-way (AB) and one way (ABm) MLR

from patients with Bruton's type of sex-linked hypogammaglobulinaemia exhibit either normal (8) or deficient (23) responses in the MLR The role of the antibody forming line of lymphoid cells in the MLR is therefore not clear

The chicken provides an attractive experimental model for studies of the cellular basis of the mixed leucocyte reactions Thus the development of the lymphoid system in this species may experimentally be manipulated by early thymectomy or bursectomy in combination with x-irradiation (14) Selective immune deficiences are produced resembling those of the human thymic aplasia (DiGeorge's syndrome) and Bruton's sex-linked hypogammaglobulinaemia (13) Furthermore, the blood group antigens B and possibly C of the chicken are also the major transplantation antigens (28) Therefore procedures based on the haemolysis or haemagglutination of chicken erythrocytes can be used to detect antibodies against transplantation antigens

In the present investigation a tissue culture technique used in this laboratory for the study of the *in vitro* secondary immune response of chicken spleen cells (1) was adapted for the mixed spleen leucocyte reaction (MLR) Some of its characteristics were investigated An attempt was made to demonstrate cells forming antibody to histocompatibility antigens using a modification of the Jerne technique (20) with chicken erythrocytes as the "target cells" for antibodies to B and other blood group antigens (25)

Finally the ability of spleen lymphoid cells from control irradiated (Cx) and hypogammaglobulinaemic bursectomized irradiated (Bx) chickens to respond in the 'one-way" and "two-way" MLR were compared

MATERIALS AND METHODS

Animals Eight week old white leghorn cockerels of the outbred DeKalb strain 161 (Munroe Hatchery, Joliet, Ill USA) and adult outbred strain 131 hens (DeKalb Agresearch, DeKalb Ill, USA) were used The former were raised from newly hatched chickens in conventional animal quarters

Bursectomy and X irradiation Surgical bursectomy was performed on strain 161 chicks on the day of hatching as previously described (27) X irradiation (600R) was performed on the same day under conditions described elsewhere (4)

Immunoglobulin assays Semiquantitative immunoglobulin assays were performed on 7 week-old chicks as previously described (4) *Severely hypogammaglobulinaemic* bursectomized irradiated chickens were selected for this investigation

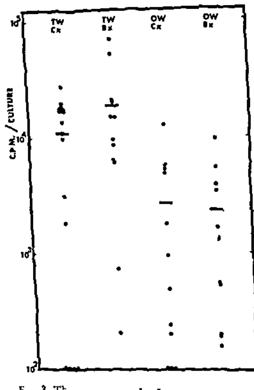
Preparation of spleen cells The preparation of spleen cell suspensions for tissue culture is described in detail elsewhere (1)

Mitomycin C treatment Spleen cells were treated with mitomycin C (Nutritional Biochemicals Corp, Cleveland, Ohio, USA) essentially as described by *Bach et al* (9) Spleen cell suspensions were prepared containing approximately 1×10^7 cells per ml of Waymouth MB 752/1 tissue culture medium supplemented with 5 per cent homologous heat inactivated chicken serum They were incubated for 20 minutes at 37 5°C with 25 ug mitomycin C per ml medium The cells were washed twice in the medium and finally resuspended to the appropriate cell concentration in the complete tissue culture medium (see below)

Tissue culture conditions The details of the tissue culture conditions are described elsewhere (1) In brief, the tissue culture medium was Waymounth's MB 752/1 containing 100 units/ml penicillin and 100 ug/ml streptomycin (Grand Island Biological Co, Grand Island N Y, USA) It was supplemented with 15 per cent (v/v) pooled fresh homologous heat inactivated (56°C for 30 minutes) chicken serum Two ml volumes, containing when not otherwise stated a total of 1×10^7 cells, were cultured in tightly stoppered 16 × 125 mm glass tubes maintained at 30° angle in a 37 5°C incubator A 5 per cent CO_2–95 per cent air atmosphere was used

Assays for antibody forming cells The indirect version (15, 32) of a technique of local haemolysis in agar gel originally described by *Jerne et al* (20) was used The details of the procedure with introduced modifications are described elsewhere (2) except that 10 per cent (v/v) chicken erythrocytes (CRBC) substituted for the sheep erythrocytes (25)

Determination of the H^3 methyl thymidine incorporation The incorporation of H^3 methyl thymidine (H^3 TdR) during a 4 hour period was taken as an indicator of the rate of the DNA synthesis and cell proliferation in the cultures One μc H^3 TdR, specific activity 6 7 c/mM (New England Nuclear Corp, Boston Mass USA) in 0 050 ml phosphate buffered saline was added per culture The incorporated radioactivity per culture was determined by liquid scintillation counting as previously described (4)



MLR. Each point represents the mean increase of the H³ TdR incorporation (c p m per culture) in one combination of allogeneic spleen cells The horizontal bars ind cate the arithmetic group mean

periments with 12 Bx and 12 Cx animals were performed

The results are summarized in Fig 3 No difference was noted between the reactivity of spleen cells from Cx and Bx animals in the MLR

In the two-way MLR the mean increase in allogeneic combinations of Cx animals was 11085 ± 2279 and that of Bx animals 18791 ± 6116 c p m per culture

In the one way MLR the corresponding increases of Cx and Bx animals were 2718 ± 1127 and 2338 ± 812, respectively

DISCUSSION

The tissue culture technique adopted for the two-way and one way mixed leucocyte reaction (MLR) between allogeneic chicken spleen lymphoid cells was similar to that previously used for the maintenance of the secondary immune response of chicken spleen cells to sheep erythrocytes (1) It permitted the study of the MLR for at least 7 days using from 2.5 to 15 × 10⁶ cells per culture

The one way MLR made use of the fact that mitomycin C, which cross links DNA and prevents its replication (33), inhibits in a practically irreversible manner the proliferative response of human peripheral blood lymphocytes in the MLR (9) It does not prevent the stimulatory effect of these cells on allogeneic lymphocytes

The mitomycin C had the same effect on the chicken spleen cells in this investigation Because the responses in the one way MLR were proportionately less than in the two way reaction it is possible that the mitomycin C treated cells caused a suppression of the reactivity of untreated allogeneic spleen cells. Such an effect has been suggested previously (18, 35)

The mixed cultures did not contain any cells forming antibody to the histocompatibility antigens carried on the CRBC used as target cells in the Jerne technique Cells forming antibody to the B and other antigens on CRBC were easily induced *in vivo* and detected by a modified Jerne technique (12, 25) Therefore the abscence of antibody forming cells in the MLR is less likely due to an inadequate assay procedure However, it cannot be excluded that cells forming antibody of a quantity or quality not detectable by the Jerne assay were present

Although the results of this investigation may indicate that the responding lymphoid cells in the MLR do not form antibody to histocompatibility antigens they do *not* exclude that they have the potential to do this Thus there is evidence that the reaction between allogeneic cells suppresses antibody formation both *in vivo* (22) and *in vitro* (26)

Because spleen lymphoid cells from hypogammaglobulinaemic bursectomized irradiated chickens responded normally both in the

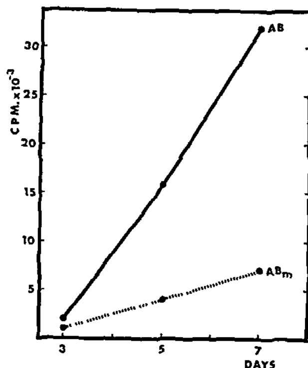


Fig 2 The effect of the culture time (days) on the increase of the H^3-TdR incorporation (c p m per culture) in the two-way (AB) and one way (ABm) MLR

The efficiency of the mitomycin C-treatment of chicken cells was therefore tested

Spleen cell cultures containing a total of 10×10^6 spleen cells, half from strain 161 (A) and half from strain 131 (B) using the combinations ABm, AAm and AmBm (m = mitomycin C-treatment) were initiated Cultures were harvested on days 3, 5 and 7

TABLE 1 *The Efficiency of the Mytomycin C-treatment in the MLR*

Combination	H^3-TdR incorporation (cpm) day 3	day 5	day 7
AB_m	1820	5420	8957
AA_m	776	1477	2261
A_mB_m	331	290	907

A and B are spleen cells from strain 161 and 131 chickens respectively, m indicates the mitomycin C-treated cells The figures represent the mean H^3-TdR incorporation (c p m) per culture

The results illustrated in Table 1 were obtained Mitomycin C-treatment completely prevented the reactivity in the MLR of chicken spleen cells during the 7-day culture period

Assay for antibody forming cells in the MLR The erythrocytes used in the Jerne assay were obtained from the chickens prior to sacrifice Two-way mixed spleen cell cultures were established from 4 chickens of strain 161, giving a total of 6 allogeneic combinations One-way cultures were initiated with mixes of spleen cells from 3 control-irradiated strain 161 chickens and mitomycin C-treated cells from a strain 131 chicken All cultures contained a total of 15×10^6 cells and were harvested after 5 days For each combination at least 2 cultures were set up Two Jerne plates were prepared from each culture (1/5 of each culture thus was assayed).

The increases of the H^3-TdR incorporation (c p m per culture) caused by allogeneic cells in the two-way and one-way MLR were 12612 ± 2960 and 3868 ± 1695, respectively No cells forming antibody specific for the blood group antigens of the stimulating lymphoid cells in the MLR could be detected in any of the cultures using erythrocytes from the spleen cell donors as target cells in the Jerne technique

The response of spleen cells from bursectomized-irradiated chickens in the MLR Two-way mixed culture using combinations of spleen cells within a group of bursectomized-irradiated (Bx) chickens and within a group of control-irradiated (Cx) chickens were established Five separate experiments using a total of 13 Bx (12 allogeneic combinations) and 15 Cx (16 allogeneic combinations) animals, were performed The cultures contained 10×10^6 cells and were harvested on day 5

One-way mixed cultures with spleen cells from Bx or Cx strain 161 chickens and mitomycin C-treated spleen cells from strain 131 chickens were established A total of 10×10^6 cells per culture was used, and all cultures were harvested on day 5 Three separate ex-

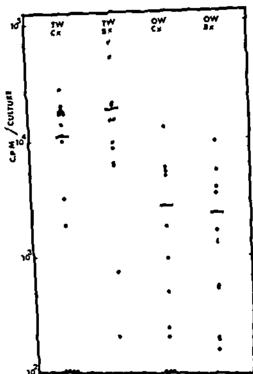


Fig 3 The reactivity of spleen cells from control irradiated (Cx) and bursectomized irradiated (Bx) chickens in the two way (TW) and one way (OW) MLR Each point represents the mean increase of the H^3 TdR incorporation (c p m per culture) in one combination of allogeneic spleen cells The horizontal bars ind cate the arithmetic group mean

periments with 12 Bx and 12 Cx animals were performed

The results are summarized in Fig 3 No difference was noted between the reactivity of spleen cells from Cx and Bx animals in the MLR

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The one way MLR made use of the fact that mitomycin C, which cross links DNA and prevents its replication (33), inhibits in a practically irreversible manner the proliferative response of human peripheral blood lymphocytes in the MLR (9) It does not prevent the stimulatory effect of these cells on allogeneic lymphocytes

The mitomycin C had the same effect on the chicken spleen cells in this investigation Because the responses in the one way MLR were proportionately less than in the two way reaction it is possible that the mitomycin C treated cells caused a suppression of the reactivity of untreated allogeneic spleen cells. Such an effect has been suggested previously (18, 35)

The mixed cultures did not contain any cells forming antibody to the histocompatibility antigens carried on the CRBC used as target cells in the Jerne technique Cells

Therefore the abscence of antibody forming cells in the MLR is less likely due to an inadequate assay procedure However, it can not be excluded that cells forming antibody of a quantity or quality not detectable by the Jerne assay were present

Although the results of this investigation may indicate that the responding lymphoid cells in the MLR do not form antibody to histocompatibility antigens they do not exclude that they have the potential to do this Thus there is evidence that the reaction between allogeneic cells suppresses antibody formation both in vivo (22) and in vitro (26)

Because spleen lymphoid cells from hypogammaglobulinaemic bursectomized irradiated chickens responded normally both in the

one-way and two-way MLR it appears that the bursa-dependent lymphoid cells, which produce immunoglobulin and specific antibody (4, 13), do not play an essential role as responding or stimulating cells or otherwise in the mixed leucocyte reaction

Lymphoid cells from thymectomized rats (29, 38) and chickens (3) are deficient in the MLR It is therefore likely that the MLR results from the interaction between allogeneic thymus-dependent lymphoid cells or between thymus dependent lymphoid cells and some other cell type, possibly macrophages (5, 10, 24)

The results of the present investigation support the "two component" concept of the lymphoid system of the chicken originally suggested by *Warner et al* (34) and redefined by *Cooper et al* (14) According to this, lymphoid cells dependent on the thymus for their ontogenetic development ("thymus-dependent") are responsible for expressions of cellular immunity *in vivo* such as the graft-versus-host reaction, homograft rejection and delayed hypersensitivity The *in vitro* reactivity to allogeneic cells apparently falls in the category of thymus-dependent functions Cells influenced by the bursa of Fabricius during the ontogeny of the lymphoid system ("bursa-dependent") produce immunoglobulin and specific antibody

This dichotomy of the lymphoid system may functionally not be complete Thus thymectomized-irradiated chickens showed significantly depressed antibody responses to several antigens (3, 14) Furthermore at least under certain conditions the *in vitro* proliferative responses of spleen cells from sheep erythrocyte immunized chickens to this antigen are impaired in both thymectomized-irradiated (3) and bursectomized-irradiated chickens (1)

I thank Dr *Raymond D A Peterson* for generous support and Mrs *Clara Mayne* for expert technical assistance throughout the investigation

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A TECHNIQUE FOR ACCESSORY CERVICAL HEART TRANSPLANTATION IN RABBITS AND RATS

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A surgical method for accessory cervical heart transplantation in rabbits and rats has been developed and the procedure is described in detail For blood vessel anastomosis the method employs an extra luminal teflon prosthesis This non suture technique is found to be easy, quick and reliable in the hands of an operator without experience in microsurgery The most frequent postoperative complication is thrombosis of the carotid artery occuring in 30 per cent of the rabbit recipients and 10–15 per cent of the rats The superficial cervical localization of the transplant is found to be advantageous for control with function of the heart, and for exact determination of endpoint of the rejection process

The first attempts at experimental heart transplantation were reported in 1905 by *Carrel & Guthrie* (2) The heart of a small dog was transplanted to the neck of a larger one and the circulation re-established by means of anastomosis to the carotid and jugular vessels in the recipient A wide variety of surgical procedures for transplantation of the dog heart have been reported since this early work, the most common method being a modification of the technique of *Mann et al* (6) Suture techniques for heterotopic heart transplantation between inbred strains of rats have been described by *Abott et al* (1) and recently *Lee et al* published the results obtained by their method (5)

This paper describes a simple and reliable nonsuture method for performing accessory cervical heart transplantation in rabbits and in inbred strains of rats

MATERIAL AND METHODS

Randomly bred New-Zealand White, Black Alaska and Brown Lop eared *rabbits* were used

The recipients were adult rabbits weighing 2 5–4 5 kg Young rabbits aged one to two months were preferred as the source of donor hearts adult rabbits were only used as donors in a few cases Anaesthesia was commenced and maintained by i v infusion of a saline Nembutal solution (0 6 mg/ml)

No antibiotic therapy was given except for local Topicin powder in the wound

Rats of two highly inbred lines, Wistar*) and Fischer§) were used in this study Fischer male rats 250–400 gm were used as recipients at first For the latest 50 transplantations the recipients have been female Wistar rats of 160 235 g The

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*) Kindly provided from the Fibiger Laboratory Landsforeningen til Kræftens bekæmpelse

§) Kindly provided from *Prof M Simonsen*, Institute of Experimental Immunology, Univ Cop

donors have been male rats of both strains weighing 150–300 gm

The rats were anaesthetized with ether throughout. Except for 150 000 I U penicillin i m. post operatively no medical treatment was given

The same *surgical technique* was used for rabbits and rats

Operations were performed under clean but not sterile conditions

Donor Preparation

The abdomen is opened by a midline incision The anterior thoracic cage is separated from the diaphragm and laterally incised to the internal mammary vessels The whole cage is then reflected upwards in order to provide maximum exposure Ligatures of silk 4–0 are placed around the superior and inferior venae cavae, the latter ligature is knotted following infusion of normal saline through the inferior vena cava into the heart (rat 5 10 ml, rabbit 15–20 ml)

A syringe is introduced into the thoracic aorta and normal saline (rat 10 ml rabbit 20 ml) is infused flushing out residual coronary blood A pair of blunt scissors is introduced into the transverse sinus and the pulmonary artery and the thoracic aorta are transsected 3 to 5 mm distal to their origin

The venae cavae distal to the ligatures are then divided and a mass ligature is placed around the pulmonary veins The vessels distal to this ligature are divided and the heart is removed from the thoracic cavity and placed in saline

Preparation of the Recipient

The recipient is anaesthetized, the fur at the anterior part of the neck is removed and a vertical incision is made in the skin at the antero-lateral side of the neck from the clavicula to the base of the jaw

The external jugular vein and the common carotid artery are dissected free and followed as far caudally as possible where a pair of soft clamps are applied The cranial end of these vessels are ligatured and severed (Fig 1), whereupon the distal free stumps are flushed free from blood and clots by means of normal saline Two holding sutures (6–0 rabbit 8–0 rat) are placed in the free end of the artery leaving the ends long The sutures are passed through the lumen of a short piece of polyethylene or teflon tubing (Fig 2) For the rabbit carotid artery a tube with an inside diameter of 1 6–1 8 mm and outside diameter of 2 2 mm is suitable, in a length of approximately 8 mm For the rat artery the tube of choice has been a 4 5 mm bit of the tip of a Medicut cannula MAR 1002 18

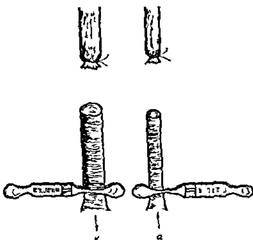


Fig 1 Common carotid artery (a) and external jugular vein (v) clamped, tied and cut

One of the sutures is passed back along the out side of the tube where a stitch is taken in the wall edge (Fig 2)

By a pull on the sutures the tube will slide on to the artery which is eased out through the end of the cuff lumen (Fig 3) The protruding artery is turned back over the cuff (which may be steadied with forceps by an assistant, if necessary) whereupon the suture is drawn tight in order to fix it, while a thin circular ligature is applied (rabbit 4–0 rat 6–0 silk) (Fig 4) The end of the holding suture is knotted as an extra precaution to prevent the vessel wall from sliding off Tubes for the jugular veins of rabbits should have a length of 8 mm and a diameter of 4–5 mm,

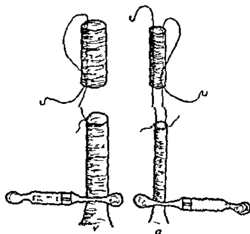


Fig 2 The teflon tubes (cuffs) being prepared for application

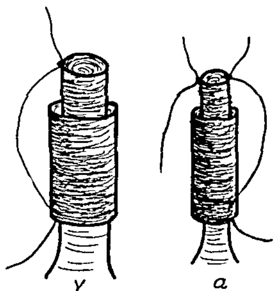


Fig 3 Tubes in position prior to 'cuffing'

and for rat veins, a length of 4 mm and a diameter in/out of 1 2/1 6 mm respectively After roughening the tube of the outside it is easily placed round the rabbit vein with the aid of fine dissecting forceps and the vein wall is turned back and tied (2–0 silk) onto this tube By means of a holdingsuture the rat vein tube is put into position (Fig 2) 6–0 silk is used as a circular ligature to hold it in place

The donor heart is transferred to the neck of the recipient The pulmonary artery is drawn over the vein cuff and a circular ligature is applied (rabbit 2 0 rat 4 0) The aorta is anastomosed to the carotid artery in a similar way (rabbit 4–0, rat 6–0 silk) (Fig 5) As an extra precaution the rabbit aorta may be sutured to the farther end of the cuff The vascular clamps are removed and the transplant regains circulation immediately

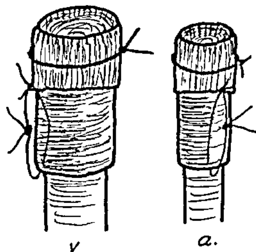


Fig 4 Artery and vein ready for vascular anastomoses

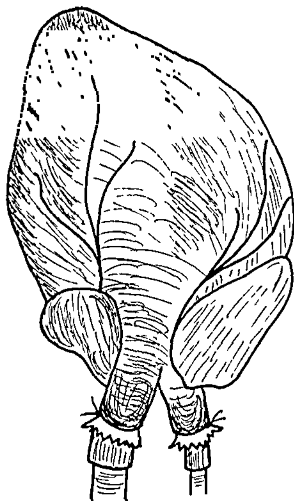
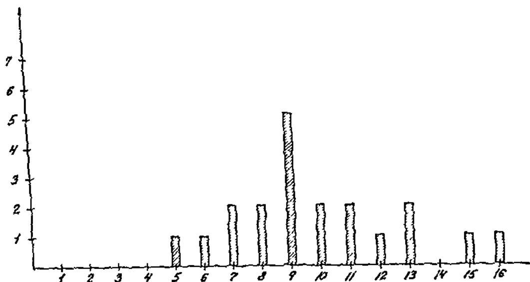


Fig 5 Donor heart brought into place The Aorta has been tied to the artery cuff, and the pulmonary artery has been secured to the vein Closure of the skin will fix the transplant in its upside down position

After a few minutes' fibrillation of the myocardium rhythmic beating begins

The entire operative procedures may be compleated within 45 minutes if preparation of the donor and recipient is done simultaneously The transplants begin to contract immediately upon being perfused with arterial blood In approximately 80 per cent of the cases the initial cardiac action consists in ventricular fibrillation which reverts spontaneously to sinus rhythm within the first few minutes Haemorrhage at vascular anastomotic sites does not normally occur In some cases the rabbit atrial auricles have been resected if overdistended with blood The heart is left in its upsidedown position and will stay in place when the skin is closed by means of a continuous suture The *mechanism of circulation* is essentially that used by *Mann et al* (6) The transplanted heart is perfused through its own coronary vessels Blood is returned to the recipient's jugular vein

TABLE 1 *Block Diagram Showing Survival Times of Heart Allotransplants in Unmodified Rabbit Recipients*



through the right atrium right ventricle, and pulmonary artery of the donor heart. In this fashion the left side of the heart is bypassed although perfusion of the left myocardium is maintained

Function of the transplant can easily be checked by inspection When rejection takes place function may be checked by palpation of beats through the skin or by ECG over the transplant in the anaesthetized animal

RESULTS

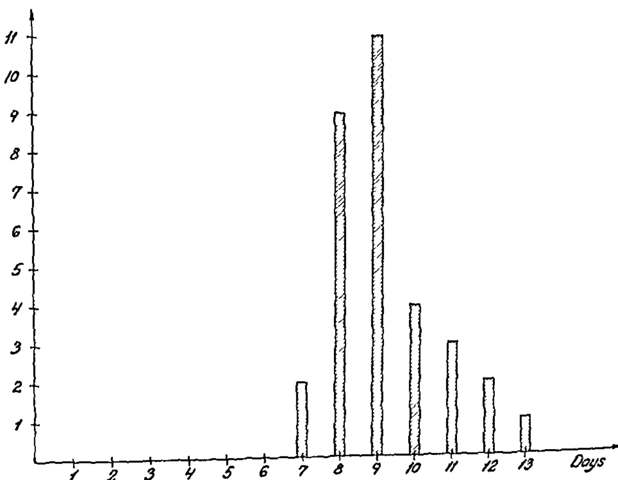
A total of 40 heterotopic heart allotransplants were performed in rabbits, and 45 allografts and 14 isografts were carried out in inbred rats On an average the time elapsing from entering the donor thoracic cavity until re-establishment of coronary circulation in the recipient was 15 minutes The uncomplicated allogeneic transplants in unmodified *rabbit* recipients (20 cases) survived with palpable beats and electrical activity for from 5 to 16 days with an average of 9.9 days (Table 1) The force of the heartbeat decreased during the last days before complete rejection, and an increase in size and tension of the transplant could be noted As soon as function had ceased totally, operative examination of the transplants was performed In uncomplicated cases, considerable coronary bloodflow was noticed at surgery dispite total loss of myocardial contractility

TABLE 2 [illegible] *Done in Unmodified recipients*

Complication	Total group	Unmodified group
Carotid artery Thrombosis	9	8
Death of recipient	2	0
Haemorrhage from injured transplant	2	0
Cardiac arrest for unknown reason	1	1
Superficial wound infection	4	3

TABLE 3 *Block Diagram Showing Survival Times of Fischer Hearts in Unmodified Wistar Recipients*

Number of transplants



Average 9 2 days

Rejection was only considered causal to functional arrest if 1) strength of the beats had ceased gradually 2) if the heart had gradually increased in size and tension and 3) if some coronary blood flow remained noticeable at surgery In 13 of the 40 successfully transplanted rabbit hearts heart beats stopped for reasons other than that of pure rejection These cases are listed as complications secondary to the operative method used (Table 2) The most frequent of these complications which occurred in approximately 30 per cent of the unmodified recipients was carotid artery thrombosis The localization of the thrombus was found in every case to be at the site of clamping during the operation Wound infection was evident in a few cases but did not affect the transplant *per se*

Rabbit recipient mortality was 5 per cent No adverse effects of the operation on the general behaviour of the animals was normally observed

The uncomplicated allogeneic transplants in *rats* underwent a clinical course similar to that of the rabbit grafts Secondary surgical complications were rare Failure of the allografts to survive until total rejection was observed in 15 per cent and in the syngeneic transplanted group 10 per cent of the transplants failed due to carotid artery thrombosis at the clamping site No infection nor other complications were observed All Wistar

recipients survived after succesfull transplantation until they were eventually sacrificed after some weeks Preliminary experiments, however, using old Fischer females as recipients suggest a postoperative mortality of approximately 25 per cent due to bronchopneumonia Five animals with isografts have been kept as longterm survivors One of these, a male Fischer rat has a well function ing transplant now 405 days after transplantation

The clinical behaviour of the isografts were identical to that of the allografts during the initial five days, but from then on the two groups differed The allotransplants started to increase in size whereas the isografts did not become larger, and some even began to diminish in size due to atrophy of the muscle of the left ventricular wall

Cardiac function could be checked very easily by inspection, except for the last few days before terminal rejection, when manual palpation had to be done to ensure presence of contractions

Survival times of allotransplanted rat hearts are shown in Table 3

DISCUSSION

According to the literature, *rabbits* do not seem to have been used in heart transplantation experiments The rabbit heart transplant was however, found to be a good model for the study of organ transplantation immunology in a randomly bred animal population The operative method described above was found to require only minimal surgical experience The idea of blood vessel anastomosis employing an extra luminal prothesis to unite the divided vessels was originally introduced by *Payr* in 1903 (7) The application of nonsuture methods for vessel anastomoses was reviewed by *Urschel & Roth* (8) In a previous paper *Heron* (3) described a modification which was found suitable for kidney vessel anastomosis in rabbit renal transplantation experiments

The fact that cessation of heart contractility in cases with complicating carotid artery thrombosis was caused by ischaemia due to absence of nourishing blood in the carotid artery, could easily be evaluated at operation by observation of blood flow in the carotid and the coronary arteries This makes it possible immediately to distinguish between the carotid thrombosis and rejection proper when contractility ceases The few cases of wound infection, though only slight, however, has resulted in the introduction of penicillin treatment and might indicate the need for cleaner surgical conditions in rabbit operations

Comments to the Rat Series

Transplantation between inbred strains of rats has many advantages one of which is the advantage of being able to have proper controls by transplantation of syngeneic tissue

A technique for heterotopic heart transplantation in the rat was first devised by *Abbott and al* in 1964 (1) By this method the donor heart was placed in the abdominal cavity and the recipient abdominal aorta and inferior vena cava were transsected and anastomoses to the aorta and pulmonary artery of the transplant was carried out by running sutures Complications were mainly due to excessive haemorrhage emerging from the aortic suture anastomoses, resulting in a considerable postoperative mortality *Lee and et* (5) recently described a method and its application to abdominal heart and lung transplantation in rats using end to side anastomosis of the aorta and right atrial opening of the transplant to the recipient aorta and vena cava by means of thin sutures In a total of 122 transplantations a mortality of 16 per cent in the first 7 days was "encountered" In a previous series of kidney grafting experiments a similar end to-side vascular anastomosis technique was used, resulting in a postoperative mortality rate of 15 per cent (out of 150 renal transplants), the major cause being due to vascular problems (4) Postoperative complications seem from this to be of equal frequency in the various materials published

A major advantage of using our method

is the superficial cervical localization which makes check on function of the transplant very quick and easy This superficial position allows an exact determination of end-point of the rejection process by palpation and ECG registration directly over the graft I feel that this method will prove useful in future organ transplantation research especially in the hands of workers who have no prior experience in microsurgery

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THE TRANSPLANTED RABBIT HEART. HISTOLOGICAL, IMMUNOFLUORESCENT AND ELECTROCARDIOGRAPHIC CHANGES

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The morphological changes have been studied in 35 ectopically allotransplanted rabbit hearts The grafts survived rejection for an average of 9.9 days in unmodified recipients and permitted serial biopsies beeing taken Generalized mononuclear cell infiltration of the myocardium, interstitial oedema, endothelial oedema and proliferation and generalized myocardial damage were the most prominent histological changes at rejection A very small number of the infiltrating cells contained cytoplasmatic γ globulins γ globulins were traced to the sarcolemma and heavy deposits could be seen intracellularly in degenerating myocardial cells Electrocardiograms showed that decline in voltage was the most reliable indication of impending cardiac rejection

Cervical transplantation of the homologous rabbit heart is a relative simple procedure that may find usefulness as an experimental technique for the study of an allografted functioning organ (7, 8) The method of circulation in these cardiac transplants is essentially that described by *Mann et al* (13), a method that may be termed a viviperfusion Because of the accessory role of the transplanted heart in the set up, this preparation can be studied without significant side effects on the recipient The present communication presents the histopathological patterns and the electrocardiographic changes in cardiac allografts in this rabbit model

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MATERIALS AND METHODS

Randomly bred rabbits of both sexes weighing 2.5–4.0 kg were used as recipients Unrelated young rabbits one to two months of age were used as donors for the *transplants* Anaesthesia was carried out using i v infusion of a saline Nembutal solution (0.6 mg/ml) Recipients of first-set *grafts* in the series were unmodified, and no systemic antibiotic therapy was given The technique of trans*plantation* has been completely described in an earlier publication (7, 8). Anastomosis of the carotid artery to the thoracic aorta of the donor heart and the jugular vein to the pulmonary artery of the transplant was carried out In this manner the heart was perfused with oxygenated blood from the host carotid artery, blood entering the coronary arteries of the transplant and returning through the coronary veins to the right atrium Blood was returned through the right ventricle and pulmonary artery of the donor heart to the recipient's jugular vein

Cardiac function following surgery was checked by simple inspection of rhythmic beating through the skin of the neck The last days before complete rejection manual palpation of the transplant was carried out to ensure function Electrocardiographic

studies were made on selected animals The electrocardiograms were taken on a Siemens Cardiomat The standard cable was used with the leads connected to 25 gauge steel needles which were inserted under the skin during anaesthesia The ECG of the grafted heart could be obtained almost free of interference from the recipient s heart when the V lead was placed under the skin directly over the transplant Operative removal and examination of the transplant was performed upon total cessation of function determined by loss of electrical activity and cases with complications (7) were excluded from the histological material Representative tissue was taken for light and fluorescence microscopy following macroscopic examination of the rejected heart

Tissue for paraffin embedding was fixed in 80 per cent ethanol and for immunofluorescent studies cryostat sections of fresh material were prepared Sections of 4 6 μ were cut and routinely stained with haematoxylin eosin and methylgreen pyronine Cryostat sections were airdried for 10 minutes and washed in buffered saline for 10 minutes FITC conjugated horse antirabbit immunoglobulins and swine antirabbit immunoglobulins were commercially produced reagents* By immunoelectrophoresis the former gave two precipitin arcs one in the IgG region the other in the IgM region Two precipitin arcs from the swine antirabbit immunoglobulin reagent were obtained one in the IgG and one in the IgM region

Sections were incubated with a few drops of labelled conjugate diluted 1 15 and left for 30 minutes in a moist chamber at 20°C After washing in buffered saline (pH 7 1) for one hour a cover glass was mounted over a drop of buffered glycerol Control sections consisted of 1) sections previously incubated with antirabbit γ globulins not bound to FITC followed by washing and staining with labelled antibodies 2) sections previously incubated with non immune serum from swine followed by washing and staining Fluorescence considered specific was considerably diminished in 1) and unaltered in 2) If the fluorescence was diminished following 2) it was registered as non specific immunologically A third control section was incubated with labelled antibodies treated with the antigens i e rabbit immunoglobulins

The sections were studied in a Leitz Ortoplan microscope with an HBO 200 Bulb as a light source Darkfield condenser was employed and photographs were taken using an Ilford FP4 film

*Centraal Laboratorium van de Bloedtransfusiedinst van Het Nederlandsche Roode Kruis and Nordic Pharmaceuticals and Diagnostics

RESULTS

The heart transplants in twenty unmodified recipients were observed to terminal, spontaneous rejection and examined following operative removal The average transplant survival time in these was found to be 9 9 days (Table 1) Fifteen other allotransplants were removed and/or biopsied at varying intervals after surgery to get information about the development of morphological changes

Fibrinous adhesions to the graft were found with surrounding serous fluid during the first couple of days following transplantation Increase in size and tension of the transplant was characteristic during the last days before complete rejection in which period contractions became irregular and less powerful Rejected transplants had increased in weight up to approximately twice the original weight and the myocardium was oedematous and swollen Remaining coronary blood flow could always be noted, despite total loss of electrical activity, when grafts were investigated at surgery following rejection Serial electrocardiograms were evaluated to detect changes which correlated with rejection Immediately after transplantation transient bradycardia and varying degrees of defective intraventricular conduction was found which reverted to normal within hours Although regular and normal QRS complexes generally persisted throughout most of the course conduction abnormalities were frequently

Fig 1 Homografted heart 1 day post transplant A linear interstitial infiltrate of mononuclear cells is present (haematoxylin and eosin ×250)

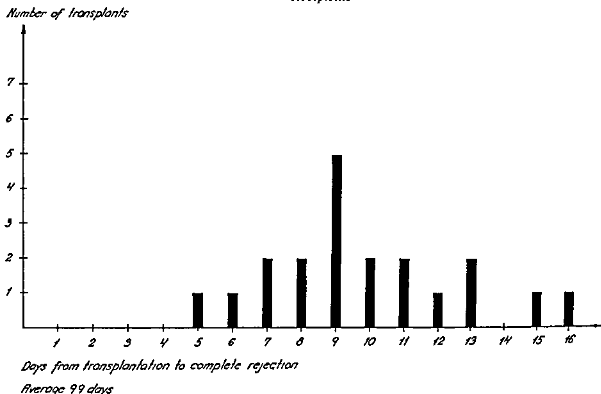
Fig 2 At 2 days following transplantation a moderately intense lymphocytic exudate surrounds a small subendocardially situated blood vessel (haematoxylin and eosin ×400)

Fig 3 Mononuclear cells with pyroninophilic cytoplasm are present in an interstitial myocardial infiltrate on day 4 (methyl green pyronine ×400)

Fig 4 Mononuclear cell infiltrate diffusely distributed and associated with degenerating myocardial fibres in a transplant at terminal rejection on day 10 (haematoxylin and eosin ×250)


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TABLE 1 *Block Diagram Showing Survival Times of Heart Allotransplants in Unmodified Rabbit Recipients*



seen in the few days preceding cessation of activity These included heart blocks, extrasystoles and ventricular arrhythmias During this period ventricular complexes were usually widened and slightly abnormal The only consistent change seen in all transplants prior to terminal rejection was a progressive reduction in QRS voltage

Microscopy

In grafts removed within 24 hours acute epicarditis was found including fibrinohaemorrhagic exudate on the surface and infiltration of polymorphonuclear leucocytes within the first millimeter of the subepicardial muscle Small haemorrhagic foci with leucocyte infiltration and degenerating myocardial cells could occasionally be found deeper in the myocardium

In transplants removed after 24–48 hours collections of mononuclear cells were observed, some with lymphocyte morphology, others looking like macrophages Cells accumulated focally around vessels (Fig 2) and others could be seen scattered interstitially among myocardial cells (Fig 1) Typical pyroninophilic cells were sometimes noted in these early infiltrates

The infiltrate during the following days (3–5) was dominated by accumulation of mononuclear cells with abundant cytoplasm large vesicular nuclei and prominent nucleoli The cells were mostly localized interstitially between myocardial fibres At this time

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Fig 5 Heavy cellular infiltration in an area with total degeneration and loss of myocardial fibres Day 13 (haematoxylin and eosin ×250)

Fig 6 Artery in a transplant on day 13 showing intimal proliferation and oedema Cell infiltration is present perivascularly (haematoxylin and eosin ×250)

Fig 7 The lumen of a medium sized myocardial artery is totally occluded by intimal cell proliferation Day 16 (haematoxylin and eosin ×400)

Fig 8 A cryostat section from a cardiac transplant rejected within 10 days (see photograph 4) stained for γ globulins Fluorescence is present within myocardial cells in a scattered and disrupted pattern (FITC conjugated swine antirabbit immunoglobulin ×250)

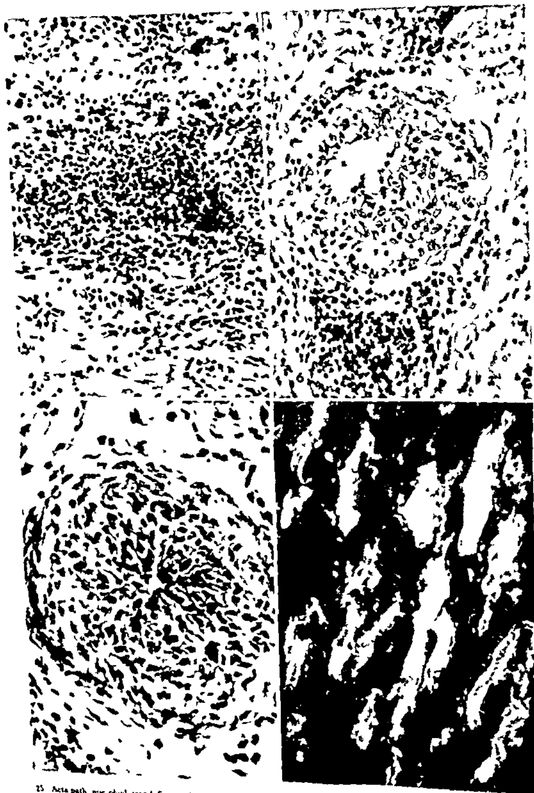
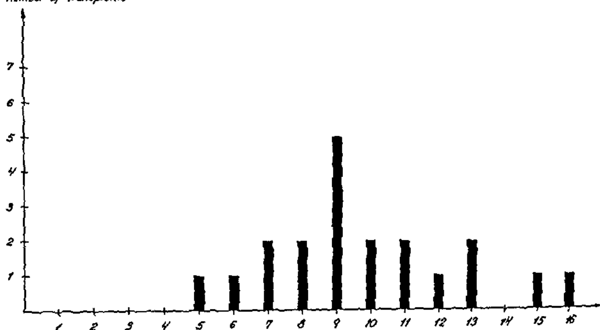

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TABLE 1 *Block Diagram Showing Survival Times of Heart Allotransplants in Unmodified Rabbit Recipients*



Days from transplantation to complete rejection

Average 9.9 days

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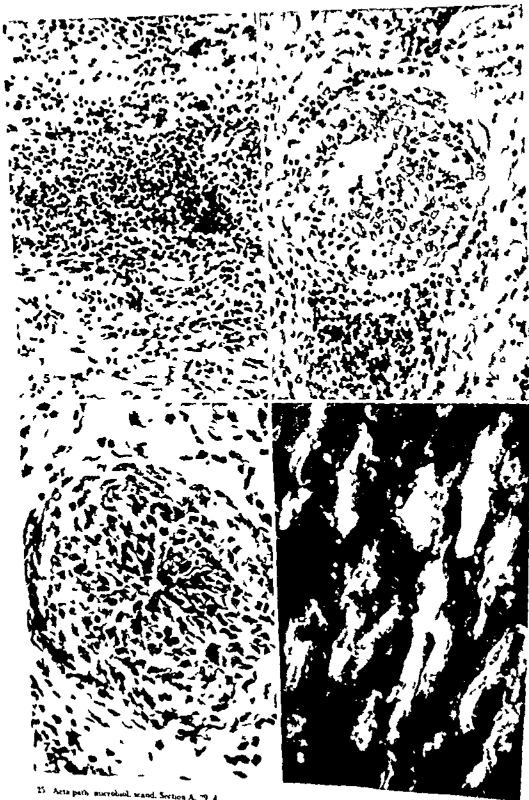
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eosinophilic leucocytes lymphocytes and mononuclear cells with pyroninophilic cyto plasm were common cell types in the myo cardium as well as in the epicardium (Fig 3)

From the 6th day on, when the transplants lost contractility densely diffuse cellular in filtration of mononuclear and also eosinophi lic cells was present (Fig 4) A varying somewhat limited number of mature plasmo cytic cells was found in the infiltrates some showing pyroninophilia Degeneration of myocardial fibres and areas of extensive myo cardial necrosis was evident with interstitial haemorrhage and fibroblastic replacement of the myocardium (Fig 5) Polymorphonuclear

peared as segmental shrinkages, sometimes together with basophilic staining or could be observed as dissolution of fibres with vacuoli zation of cytoplasm leaving only the cell out lines In some cases a sharply demarcated necrotic myocardial area was noted concen trically around the left ventricle

The left ventricle and atrium contained organizing thrombi secondary to stasis of blood in these chambers Mural thrombosis to a greater or lesser extent was occasionally observed in the right ventricle Endothelial cells of the endocardium showed proliferation and a severe mononuclear cell infiltration was present subendocardially

Vascular changes except for swelling of endothelial cells were not prominent in the early periods following transplantation where as advanced rejection was accompanied by pronounced alterations Oedema of the ad ventitia and intima of the greater vessels was evident (Fig 6) and proliferation of endothe lial cells varied in extent to complete oblitera tion of the vascular lumen (Fig 7)

Fluorescent antibody studies

It was possible by immunofluorescent tech niques to demonstrate a few typical patterns of γ globulin localization in the homografts during rejection

The most prominent of these was faint localization of γ globulins to the sarcolemma of degenerating myocardial cells followed by intense fluorescence intracellularly in a scat tered and disrupted pattern (Fig 8)

Very few mononuclear cells containing cytoplasmatic deposits of γ globulins were observed in the rejected transplants On an average 5 10 cells were seen per myocardial cross section, some containing Ig M others Ig G They generally appeared in small clusters

Vascular localization of immunoglobulins was very rarely present, but if observed they were localized diffusely throughout all layers of the vessel wall

DISCUSSION

The author has not found any previous re ports concerning heart allotransplants in rab bits The present study supports the general assumption that homografted organs trans planted across a major histocompatibility barrier are rejected acutely in unmodified recipients and with similar morphology with in different animal species with only few exceptions (2, 15) Heterotopic and ortho topic cardiac transplants in dogs have been studied extensively (3, 4 10 12 14 16) and more recently rats have been the species of choice for grafting experiments (1 11) mostly because of the existence of inbred strains offering considerable advantages

The findings by electrocardiographic stud ies of rabbit transplants support the conclu sion made by others on the basis of investiga tions using dogs rats and man that the only ECG parameter giving reliable indication of impending rejection is a decline in voltage *Lee and al* however (11) who studied the allotransplanted rat heart and lung prepara tion did not agree on this point as they found that elevation of ST segments was the most prominent ECG feature of rejection in their experiments

The presented histological data suggest that the introduction of a cardiac allograft into a rabbit leads to the induction of cell mediated immunity characterized by a mononuclear

eosinophilic leucocytes, lymphocytes and mononuclear cells with pyroninophilic cytoplasm were common cell types in the myocardium as well as in the epicardium (Fig 3)

From the 6th day on, when the transplants lost contractility, densely diffuse cellular infiltration of mononuclear and also eosinophilic cells was present (Fig 4) A varying, somewhat limited, number of mature plasmocytic cells was found in the infiltrates, some showing pyroninophilia Degeneration of myocardial fibres and areas of extensive myocardial necrosis was evident, with interstitial haemorrhage and fibroblastic replacement of the myocardium (Fig 5) Polymorphonuclear cells accumulated around necrotic myocardial cells Degeneration of myocardial cells appeared as segmental shrinkages, sometimes together with basophilic staining or could be observed as dissolution of fibres with vacuolization of cytoplasm, leaving only the cell outlines In some cases a sharply demarcated necrotic myocardial area was noted concentrically around the left ventricle

The left ventricle and atrium contained organizing thrombi secondary to stasis of blood in these chambers Mural thrombosis to a greater or lesser extent was occasionally observed in the right ventricle Endothelial cells of the endocardium showed proliferation and a severe mononuclear cell infiltration was present subendocardially

Vascular changes, except for swelling of endothelial cells, were not prominent in the early periods following transplantation whereas advanced rejection was accompanied by pronounced alterations Oedema of the adventitia and intima of the greater vessels was evident (Fig 6) and poliferation of endothelial cells varied in extent to complete obliteration of the vascular lumen (Fig 7)

Fluorescent antibody studies

It was possible by immunofluorescent techniques to demonstrate a few typical patterns of γ globulin localization in the homografts during rejection

The most prominent of these was faint localization of γ-globulins to the sarcolemma of degenerating myocardial cells followed by intense fluorescence intracellularly in a scattered and disrupted pattern (Fig 8)

Very few mononuclear cells containing cytoplasmatic deposits of γ globulins were observed in the rejected transplants On an average 5-10 cells were seen per myocardial cross section, some containing Ig M, others Ig G They generally appeared in small clusters

Vascular localization of immunoglobulins was very rarely present, but if observed, they were localized diffusely throughout all layers of the vessel wall

DISCUSSION

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ADDISON'S ADRENALITIS

Studies on Diffuse Lymphocytic Adrenalitis (Idiopathic Addison's Disease) and Focal Lymphocytic Infiltration in a Control Material

MICHAEL PETRI and JORN NERUP

The University Institute of Pathological Anatomy, Copenhagen (Heads G Teilum and E Jensen) and Medical Department A Copenhagen University Hospital, Rigshospitalet, Copenhagen (Head K Brochner Mortensen)

Based on review of the literature and ten cases of our own it is concluded that the specific adrenal lesion in patients with *idiopathic Addison's disease* is a diffuse lymphocytic adrenalitis It is proposed to substitute for the term idiopathic Addison's disease the name *Addison's adrenalitis* since the disease has clinical, immunological and patho anatomical features of its own Adrenal glands from two autopsy series of 413 and 161 cases were investigated for the occurrence of *focal lymphocytic adrenalitis* Small numbers of lymphocytes were found in 15 per cent and 18 6 per cent respectively Lymphocytic infiltrates which to some extent resembled the infiltrates in *focal thyroiditis* were found in only 2 4 per cent and 1 8 per cent in the two series These infiltrates were also very small and the existence of a significant focal lymphocytic adrenalitis seems questionable

In his original paper On the constitutional and local effects of disease of the suprarenal capsules *Thomas Addison* (1855) included a case in which the adrenal glands appeared exceedingly small and atrofied It has now for many years been well established that in cases of idiopathic Addison's disease the adrenals are very small and often very difficult to localize during autopsy Microscopy reveals a completely disturbed architechture of the cortex characterized by massive loss of epithelial cells and infiltration with mononuclear cells This condition has been described in the literature under a variety of names, such as idiopathic adrenal atrophy, cytolytic necrosis, cytotoxic cortical necrosis, cytotoxic contraction, adrenocortical contraction and even simple atrophy In recent years it has been suggested that autoimmune mechanisms may be instrumental in the development of idiopathic Addison's disease This assumption is based upon various histopathological, serological experimental and clinical observations (*Nerup et al* 1966, *Irvine et al* 1967 and *Nerup* & *Bendixen* 1969)

Taking this hypothesis into consideration, the purposes of the present communication were

1) to review the pathology of the adrenal gland in idiopathic Addison's disease and

2) to look for the possible existence of a focal type of this lesion

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The valuable help by Dr *Harald Olesen* in interpreting the histological material is gratefully acknowledged

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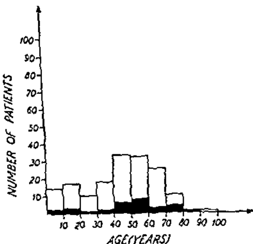


Fig 1 B Group 2 b Age distribution Cases with lymphocytic infiltration in black

complete histological investigation performed in cases of sudden unexpected or accidental death in which no satisfactory cause of death was found in the autopsy room Fixation and staining as above

Age distribution of both series is shown in Figs 1 A and 1 B

In the series 2a) and 2b) the mononuclear cell infiltrates were graded arbitrarily according to size in three grades 1+, 2+ and 3+ (Figs 2 to 5)

RESULTS

In the 10 cases of idiopathic Addison's disease the microscopical picture was uniform and consistent (Figs 6–9) The capsule presented itself thickned, the cortex was narrow and infiltrated with mononuclear cells whereas the medulla was intact, although with some mononuclear infiltration In higher magnification all three zones of the cortex appeared to be destroyed with only few adrenocortical cells left In some cases nests of cells and small regeneratory nodules could be found Some of the cells might be necrotic and many were pleomorphic or enlarged with increased acidophilia The majority of the inflammatory cells were small lymphocytes, but some plasma cells were also demonstrable No germinal centres were present Fibrosis was demonstrable but did not seem to be of a cicatricial type In fact, the process

TABLE 2 group 2 a *Lymphocytic Infiltrates Graded According to Size*

Type of infiltrate	Males n = 254	Females n = 159	Total n = 413
1+	27(10 6 %)	11(6 9 %)	38(9 2 %)
2+	12(4 7 %)	2(1 2 %)	14(3 4 %)
3+	4(1 5 %)	6(3 7 %)	10(2 4 %)
Total	43(16 8 %)	19(11 8 %)	62(15 0 %)

seemed sometimes to respect the original fibrous skeleton of the cortex In other cases the fibrous cortical zone was extremely thin and reduced to a narrow band Thus the essential lesions seemed to be a diffuse lymphocytic infiltration and a selective destruction of the epithelial cells of the cortex

In the two autopsy series lymphocytic infiltration was noted with the same frequency in group 2 a and group 2 b, 15 per cent and 18 6 per cent respectively The frequency graded according to the size of the infiltrates is shown in Tables 2 and 3 It appeared that in all cases the total number of mononuclear cells was very small, constituting an almost negligible part of the whole section Further, in cases graded as 1+ and 2+ the collections of lymphocytes had a rather even circumference and contained few cortical cells In the 3+ cases the lymphocytic infiltrates, while constituting also only a very insignificant part of the gland, were larger, had a more diffuse outline and contained cortical cells, some of which were pleomorphic

In group 2 a) infiltrations coccurred more

TABLE 3 group 2 b *Lymphocytic Infiltrates Graded According to Size*

Type of infiltrate	Males n = 87	Females n = 74	Total n = 161
1+	11(12 7 %)	13(17 6 %)	24(15 %)
2+	2(2 2 %)	1(1 4 %)	3(1 8 %)
3+	1(1 1 %)	2(2 8 %)	3(1 8 %)
Total	14(16 %)	16(21 8 %)	30(18 6 %)

TABLE 1 *Clinical Summary of 10 Patients with Addison's Adrenalitis*

Sex	Age	
Females	9	Symptoms for 3 years Addison's disease diagnosed at 7 years
	12	Symptoms (skin pigmentation, asthenia) for 5 years Addison's disease suspected Dies in Addisonian crisis, caused by enteritis (salmonellosis). These two cases have been published by *Bie* (1956)
	21	Addison's disease diagnosed at 12 years Graves's disease 17 years old, treated by partial thyroidectomy Dies suddenly
	43	Diabetes mellitus for a number of years Addison's disease diagnosed at 37 years Death caused by sepsis
	60	Well until 6 days before death Autopsy showed diffuse adrenalitis and Hashimoto's thyroiditis
	65	Addison's disease never diagnosed Amputation of right lower extremity (reticulosarcoma) 13 years previously Besides diffuse adrenalitis, obesity and ependymoma are the main autopsy findings
	70	Hyperthyroidism 1946, treated by X ray Addison's disease diagnosed 1956 Dies 1968
	72	Symptoms one year before diagnosis Treatment for Addison's disease is started but the patient dies from cardiac incompensation (severe atherosclerosis and myocardial fibrosis) Latent pernicious anaemia Parietal cell and adrenocortical cell antibody demonstrated Autopsy showed also marked focal thyroiditis
Males	16	Weight loss asthenia for app 6 months after throat infection Dies in Addisonian crisis after severe vomiting for 2 days
	48	Addison's disease suspected for 9 months prior to death in 1946 Previously well No pigmentation

MATERIALS AND METHODS

The material comprises a total of adrenal glands from 657 patients

1) Adrenal glands from 10 patients with idiopathic Addison's disease A clinical summary of these cases is given in Table 1

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a) 488 patients coming to autopsy in the University Institute of Pathological Anatomy Copenhagen, during 1966 In 413 a good paraffin section was available from one or both adrenal glands The sections from the glands were removed for microscopy by routine at the autopsy table and the site chosen at random according to the judgment of the pathologist in charge All sections were fixed in formol and stained by haematoxylin and eosin or by van Gieson

b) 161 adrenal glands from autopsies performed during the years 1964 1968 at the University Institute of Forensic Medicine Copenhagen The adrenal sections were taken mainly as part of a

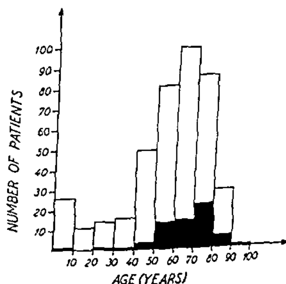


Fig 1 A Group 2a Age distribution Cases with lymphocytic infiltration in black

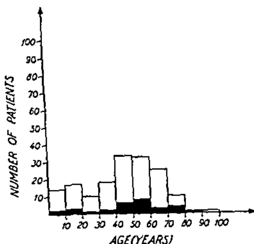


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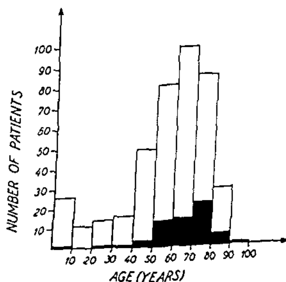


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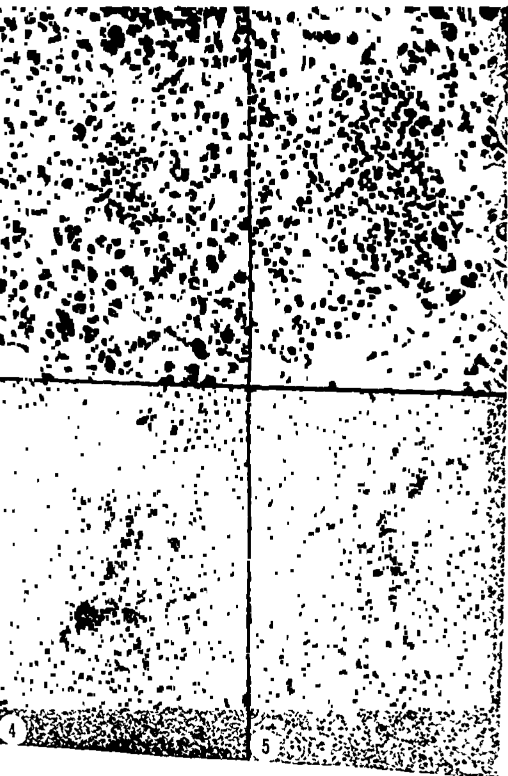

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TABLE 4 *Clinical Diagnoses of Patients with 3+ Infiltrates*

Sex	Age	Diagnoses
Males	47	Rheumatic heart disease Death 5 days after mitral valvulotomy
	57	Automobile accident
	74	Chronic bronchitis, cor pulmonale
	81	Gastric carcinoma, postoperative intraperitoneal abscess (E coli)
	82	Diabetes mellitus, myocardial infarct
Females	44	Found at home unconscious Mild myocardial fibrosis
	54	Rheumatic heart disease (mitral and aortic stenosis) Intracerebral haemorrhages
	56	Diabetes mellitus, myocardial infarct
	65	Rheumatoid arthritis, thrombocytopenia sepsis
	69	Obesity Severe burns
	71	Diabetes mellitus, myocardial infarct
	72	Myocardial infarct,
	77	Rheumatic heart disease (mitral stenosis), hepatitis, obesity, pulmonary embolus

frequently in males than in females, but the difference was not statistically significant ($p > 0.1$)

Group 2 b) had more females positive than males, but again the difference was not statistically significant ($p > 0.1$) The infiltrates occurred in all age groups as seen in Fig 1 A and 1 B

Grade 3+ infiltrates were found in only 13 cases and occurred in group 2 a) and 2 b) in 2 4 per cent and 1 8 per cent, respectively Table 4 shows the clinical diagnoses in these cases, which were predominantly females

DISCUSSION

The adrenal lesions in the cases of idiopathic Addison's disease described above are in agreement with the findings of several other authors (*Guttman* 1930, *Wells* 1930, *Susman* 1930, *Duffin* 1943, *Friedmann* 1948, *Symington* 1969 and *Maisey & Stevens* 1970)

Symington (1969) states that the lesion is specific for this type of Addison's disease

This picture, viz lymphocytic infiltration and disappearance of cortical cells, is compatible with the concept that the lesion might be the ultimate result of a specific direct reaction between immunologically competent cells and antigenic components in the adreno cortical cells Although no germinal centres were demonstrated in this series, these findings are analogous to those in Hashimoto's thyroiditis, and we therefore find it reasonable to propose the term *diffuse lymphocytic adrenalitis* as a name of this condition, instead of the above mentioned variety of non descriptive names hitherto used in the literature

Thomas Addison (1855) himself was aware of the possibility of a direct toxic ef

Fig 2 Grade 1+ Size and composition of cellular infiltrate (350 ×)

Fig 3 Grade 2+ Similar infiltrate of somewhat larger size (350 ×)

Fig 4 Grade 3+ Larger infiltrates in the deeper layers of the cortex (140 ×)

Fig 5 Grade 3+ Infiltrate at the cortico-medullary junction (140 ×)

fect of the infiltrating cells on the parenchymal cells since he wrote in his original paper the following comment to the case in his own series which presented "exceedingly small and atrofied' adrenal glands at autopsy 'It is, moreover of some significance and importance to observe that in the present instance, the diseased condition of the supra renal capsules did not result, as usual, from a deposit either of a strumous or malignant character, but appears rather to have been occasioned by an actual inflammation, that inflammation having destroyed the integrity of the organs and finally led to their contraction and atrophy

For this reason, in analogy to the eponym Hashimoto s thyroiditis corresponding to the chronic, diffuse lymphocytic thyroiditis we find it tempting to substitute for the term idiopathic Addison's disease the name *Addison's adrenalitis* since this condition has clinical characteristics immunological features and a patho anatomical picture of its own

In the two groups of autopsies clusters of lymphocytes were found with a frequency of 15–19 per cent All of them were small and their number surprisingly low not exceeding 10 in the sections studied Thus the total number of lymphocytes in all cases was small Focal lymphocytic thyroiditis has been found to occur with similar frequences in large groups of autopsies of patients without clinical signs of thyroid disease (*Goudie et al* 1959, *Williams* & *Doniach* 1962, *Bastenie et al* 1967 and *Halberg et al* 1968) The number of lymphocytes, and the number of focal infiltrates in these cases, however, far exceeds what was found in the adrenals in the present series It should be mentioned that the autopsy material reported by the last named authors (*Halberg et al* 1968) is identical with group 2 a of the material in this work.

Lymphocytic infiltrations in the adrenals of the present series occurred equally frequent in both sexes, whereas focal thyroiditis shows a predominance in the female sex The incidence of focal thyroiditis increases with age This could not be convincingly demonstrated with lymphocytic infiltrations in the adrenals, the frequency of which follows the age distribution of the whole material (Fig 1)

The 3+ infiltrates, being the only infiltrates which to some extent resembled the infiltrates in focal thyroiditis, occurred in only 2 4 per cent and 1 8 per cent of the two groups in the present series Furthermore, the size of the infiltrates was not very impressive so the total number of lymphocytes was small Consequently it does not seem established with certainty that these findings really represent what could be called a focal lymphocytic adrenalitis though this possibility cannot be totally disregarded Transitional forms between the 3+ and the diffuse lymphocytic adrenalitis (Addison's adrenalitis) have to our knowledge not been published and were not found in the present material Until such cases are reported the existence of a significant focal adrenalitis seems questionable This concept is supported by experienced forensic pathologists (*Voigt* 1966, 1970) Reports of such cases would be of great interest

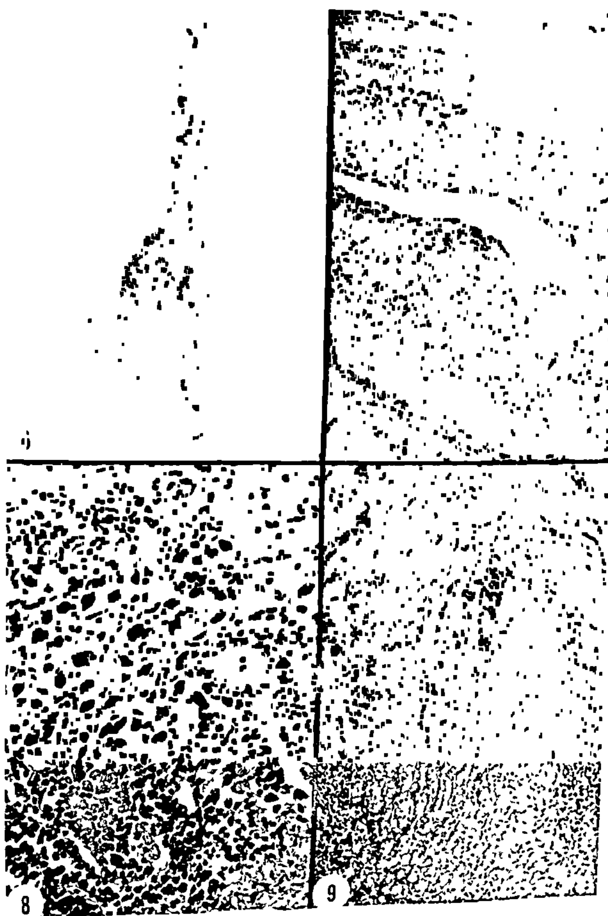
The existence of thyroid microsomal antibody and thyroglobulin antibody in titres >250 in sera from patients without Hashimoto s disease reflects the occurrence of focal lymphocytic thyroiditis (*Goudie et al* 1959 *Bastenie et al* 1967, *Halberg et al* 1968) Thus the rarity of 3+ lymphocytic infiltrations is in accordance with the very rare oc

Fig 6 Diffuse lymphocytic adrenalitis Outside the intact adrenal medulla the thin fibrous cortex is seen beneath the thickened capsule (7 ×)

Fig 7 Lymphocytic infiltration in the medulla and fibrous cortical zone (84 ×)

Fig 8 Closer view of typical cortical infiltrate with lymphocytes and pleomorphic cortical cells (350 ×)

Fig 9 The trabecular pattern is maintained beneath the thickened capsule Blood filled sinusoidal spaces are separated by columns of mononuclear cells (140 ×)


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COMPLETENESS AND RELIABILITY OF LUNG CANCER REGISTRATION IN THE SWEDISH CANCER REGISTRY

STURE LARSSON

The Department of Thoracic Surgery, Sahlgren's Hospital, University of Gothenburg, Gothenburg, Sweden, and from the Swedish Cancer Registry, National Board of Health and Welfare, Stockholm, Sweden

A study of the efficiency of registration in the Swedish Cancer Registry of primary lung cancer during the period 1959 1965 is presented The investigation was designed as follows

1) analysis of the Registry data with regard to accuracy and basis of diagnosis, and the possibility of over registration,
2) checking of a clinical series with respect to completeness in reporting, and
3) study of the reliability of histological types as recorded in the Registry

The data used for these studies are described

Over registration had occurred in 1 6 per cent due to duplicate registration, inclusion of non malignant cases and cases of neoplastic disease other than lung cancer Errors in registration of sex and residence were insignificant The causes of the errors are discussed as well as preventive measures According to the Registry the diagnosis had been based on positive histology in 94 7 per cent and on cytology only in 2 5 per cent In the final revised series positive histology had been obtained in 96 8 per cent and cytology only in 1 1 per cent. The frequency of microscopical confirmation of the diagnosis was thus very high and yet the completeness in reporting seems to have been very satisfactory Every patient in the checked sample had been reported, but errors as to site classification had deprived site 162 1 of 3 9 per cent of the patients and 1 5 per cent had incorrectly been recorded in the benign lists The causes of errors as to site classification and coding of malignancy are discussed Registration of histological types of primary lung cancer *in* the Registry seems not to produce the desired results The recorded histological diagnosis epidermoid carcinoma was correct in 77 per cent of the cases, adenocarcinoma in 79 per cent, and anaplastic carcinoma in only 46 per cent as per re-classification The causes of these discrepancies are discussed When the series was divided into only two histological groups according to *Kreyberg* consistency with re classification was observed in 84 per cent in group I and 86 per cent in group II and this is considered rather satisfactory

Cancer Registries have now been in operation for several years in many countries, making morbidity lists available for statistical cancer research A survey of the literature, however, disclosed very little on the completeness and reliability of Registry data A study not presented here of the contribution of surgery to treatment of carcinoma of the lung was designed Such a study should be based on complete and reliable data for a geographically defined region and only include patients with cancer of the lung specified as primary

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currence of antiadrenal antibody in sera from control materials and patients without Addison's disease (*Nerup* 1971) It is of interest to observe that 4 out of 13 patients with 3+ infiltrations in the adrenals of the present series had a diagnosis of diabetes mellitus, since it is known that humoral and/or cellular anti adrenal hypersensitivity occurs more frequently in diabetics than in non diabetic controls (*Nerup* & *Bendixen* 1969, *Nerup et al* 1970)

We gratefully acknowledge the material from cases of Addison's adrenalitis which was generously lent us by several Danish pathologists For this we want to thank Dr *W Kiær*, Dr *J V Thorborg*, Professor *Steen Olsen*, Dr *J Vesterdal Jørgensen* and Dr *K E Sjølin*

We also thank Dr *J Voigt*, Head of the department of pathology of the University Institute of Forensic Medicine, for his support and for permission to examine a series of legal autopsies

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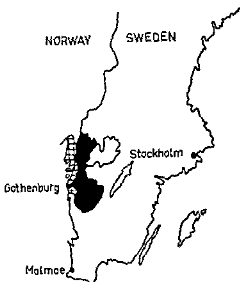


Fig 1 Southern part of Sweden with 'Goteborgs och Bohus lan' and 'Älvsborgs lan' (hatched and black respectively)

cases classified under s te 162 1 (bronchus and lung, primary) and 988 of these patients were recorded as residents in the two-district region This population is analysed with respect to accuracy and basis of diagnosis It is also checked with regard to duplication and errors in the registration of sex and place of residence

A diagnosis based on autopsy with histological examination has been considered proven (497 cases) A diagnosis primarily founded on histological examination of biopsy specimens from the primary tumour has been regarded as definitely confirmed if a fatal outcome of the disease was known to the Registry (183 cases) When the diagnosis was either histologically confirmed, but the course of the disease unknown (256 cases), or it was not histologically confirmed (52 cases), the patient has been traced and re examined The patients who were still alive (1970) have been followed for 5 years or more In all these 308 cases the diagnosis has been checked and confirmed or altered with consideration taken to all new facts including autopsy findings in a considerable proportion

Completeness in Reporting

The series used for investigation of the completeness in reporting consists of patients seen in the clinic of thoracic surgery during 1959 through 1965 Patients with suspected or verified carcinoma of the lung were primarily included, in all 302 cases, 252 of whom had been operated on The remaining 150 cases had been remitted to the clinic only for diagnostic investigation (consultation cases) Follow-up studies of these patients including re-examination of all histological slides and analysis of autopsy findings led to a final conclusion that 137 of them had primary carcinoma of the lung though never histologically confirmed in 2.9 per cent These cases have not been reported to the Registry by physicians in the clinic of thoracic surgery, but they should have been notified by the consulting physicians working in many hospitals throughout the area when the disease was diagnosed The final series, consisting of 389 cases which thus should have been reported, is checked against the lists of the Cancer Registry

Histological Type Registration

The Registry records the reported histological type of carcinoma of the lung into one of 4 categories 1) squamous carcinoma, 2) anaplastic and unspecified carcinoma, 3) adenocarcinoma, and 4) bronchiolar carcinoma. The "anaplastic" group includes large and small cell carcinoma, oat-cell carcinoma and such tumours where the pathologist only reports the diagnosis as "cancer of the lung" Adenoma of the bronchus are to be reported but they are recorded on a separate list of benign tumours (not included in the main list) The registration of histological type is based on reports from many different pathologists who often had to base their diagnosis on a small biopsy specimen If subsequent examination of the whole tumour removed at operation or autopsy has led to a more detailed diagnosis this has only rarely been notified

of the University Pathology Department and this writer All available histological data in a series of 541 consecutive patients with carcinoma of the lung seen in the clinic of thoracic surgery in Gothenburg during 1959 through 1967 was re-examined and the tumours classified according to the histological typing of lung tumours proposed by WHO Reference Centre (*WHO* 1967). In about 60 per cent of the cases, material derived from lung resections were avaiable Special staining methods for mucin like substances and keratin have been used in a part of the material The patients have been traced in the files of the Registry and the recorded histological diagnosis compared with the diagnosis according to the re-classification

RESULTS

At the examination of the Registry material based on the annual lists (988 cases) it was found as to

Therefore this study, undertaken to test the efficiency of registration of cancer of the lung, is confined to two districts of Sweden because this area is served by only one clinic of thoracic surgery, and includes only patients recorded under category 162 1 (bronchus and lung, primary) The investigation presented here includes 1) an analysis of the Registry data with regard to accuracy and basis of diagnosis, and the possibility of over-registration and 2) a check of a clinical series of patients with carcinoma of the lung with respect to completeness in reporting to the Registry and 3) an examination of the accuracy of recorded histological type of tumour making use of an another clinical series which previously had been subjected to a study of histological typing of lung carcinoma

The Swedish Cancer Registry

The Swedish Cancer Registry has been in operation since January 1st, 1958 The Registry's data are based on compulsory reports from all physicians at in- and out-patient departments in hospitals, asylums and other establishments for medical treatment, under public or private administration Private practitioners are excluded from compulsory reporting Pathologists, cytologists and forensic autopsists give independent compulsory reports of every diagnosis made on surgical biopsies, cytological specimens, and autopsies (including forensic necropsies)

The reporting has to be done the first time the tumour is diagnosed Repeated notification is not required at readmission into the same, or at admission into another, hospital If subsequent examination, however, leads to an alteration or completion of the first reported diagnosis this has to be notified An autopsy finding in discordance with an earlier reported diagnosis should also be reported

The Registry files incoming reports after a system which is based on the citizen's so-called personal number which contains 9 digits indicating date of birth and a serial number specific for each individual

If it can be concluded from a report that a notification from another source of information is lacking the Registry makes inquires This system makes it probable that most cases with microscopic confirmation are fully reported but does not garantee the completeness of the registration of cases with another basis of diagnosis In a number of cases the first report — especially if it pertains to an autopsy finding — reaches the Registry with considerable delay On the other hand, in a number of cases the diagnosis is altered several years after the first report Although the Registry does not compute its data until at least two years later a few cases have to be added to and a few cases withdrawn from the yearly lists on which the annual reports are based The Registry believes, however that the lists for the period 1959–1965 by now (1970) are as complete as possible During this period the Registry has not used death certificates for registration purposes

The Registry has used the following system for recording the basis of diagnosis

1 Clinical examination only
2 X-ray diagnosis
3 Histological examination of surgical or biopsy material
4 Autopsy (with histological examination)
5 Cytological diagnosis
6 Gross examination at operation or autopsy

MATERIAL AND METHODS

The Selected Area and Its Population

A geographically defined region consisting of two districts — Goteborgs och Bohus lan and Alvsborgs lan in the south western part of Sweden has been selected for this study (Fig 1) The total area is 16 666 square kilometres i e about 4 per cent of the whole country The population on November 1st 1965, was a little over one million (1 086 692) about 1/7 of the whole population The biggest city in the region is Gothenburg with 421 283 inhabitants in 1965

Accuracy and Basis of Diagnosis

During the period 1959 through 1965 the yearly lists covering the whole of Sweden included 8129

found in the files in a hospital during the follow up study and this patient has also been included in the final unselected series of patients which thus amounted to 998 cases The distribution of this series on the basis of diagnosis after the follow up study was

	%
Positive histology	*96 8*
Autopsy without histology	*0 1*
Positive cytology	1 1
X ray diagnosis	2 0

By comparison with the recorded basis of diagnosis in the Registry the frequency of positive histology rose from *94 7* to *96 8* per *cent, mainly due to a reduction* of the group with positive cytology from 2 5 to 1 1 per cent The recorded basis of diagnosis was thus correct in a very high percentage

The percentage distribution of bases of diagnosis in the final material from the area *is shown in* detail by sex and residence in Table 1 As *can be seen there are only small* differences between males and females or urban and rural areas Positive histology and/or cytology only had been obtained in 98 per cent among both sexes Autopsy had been performed in at least 51 8 per cent In the group with positive histology based on examination of biopsy *specimens a considerable* part had been submitted to autopsy, but in these cases the recorded basis of diagnosis had not been altered As stated before reporting of autopsy is not required when the patient has been notified previously

Comparison between the histological types recorded in the Cancer *Registry and the types* diagnosed through re-classification is shown in Table 2 A few cases with bronchiolar cell carcinoma have been included in the group adenocarcinoma The group of anaplastic carcinoma decreased *from* 43 to about *27* per cent after re classification *whereas the group* of epidermoid carcinoma increased from 41 5 to 47 5 per cent and the group of adeno carcinoma from 12 to 21 per cent As regards the cases with epidermoid carcinoma as re corded in the Registry, 77 per cent tallied at

TABLE 1 *Evidence of Malignancy in 998 Patients with Primary Lung Carcinoma by Sex and Place of Residence Given as Percentage of Row Totals*

Sex Residence	Total no	Basis of diagnosis* 1	2	3	4	5	6
Male	754	*0*	2	47 2	*50 0*	*0 7*	*0 1*
Female	244	0	2	38 5	57	2 5	0
Gothenburg	596	0	1 3	45 3	53	0 3	0
Other towns	196	0	3 1	46 4	49	1 0	0 5
Rural areas	206	*0*	2 9	43 2	*50 5*	*3 4*	*0*
Total	998	0	2 0	45 1	51 7	1 1	0 1

* Symbols explained on page 390

the re-classification, among those with adenocarcinoma 79 per cent, and among those with anaplastic carcinoma only 46 per cent Seven cases filed in the Registry as anaplastic carcinoma *or adenocarcinoma* were re classified as bronchial adenoma *Kreyberg, 1962,* has proposed that it is suitable in certain studies to divide the lung cancer types into only two groups Epidermoid carcinoma and anaplastic lesions have similar predominance of males (Group 1) and adenocarcinoma and bron*chial adenoma* have nearly equal representation of the two sexes (Group 2) When the present series was divided according *to this* grouping, 84 per cent of the cases in group 1 tallied at the re classification and 86 per cent in group 2

DISCUSSION

Conditions in the part of Sweden selected for this study would seem to favour detection and reporting of a very high proportion of *cases of primary lung cancer arising in* the population Modern medical facilities are readily accessible and the *organization* of histological and cytological *diagnostics is well* developed Analysis of the basis of *diagnosis* showed that histological and/or cytological confirmation had been obtained in 98 per *cent in the finally* revised series and it was *only slightly higher in* Gothenburg (98 6 per cent) than in other *towns (96 4* per cent) or rural areas (97 1 per cent) The proportion of such cases is the most *commonly used*

1) accuracy of diagnosis
   a) 5 non malignant cases erroneously diagnosed and reported as primary lung cancer None of them had received any treatment Two patients had been reported because of positive cytology and 3 because of X ray diagnosis,
   b) 4 patients with neoplastic disease but incorrectly diagnosed and reported primary site according to autopsy

Alteration of the diagnosis had not been reported,

2) duplicate registration 7 patients had been recorded twice,
3) sex registration 2 male patients had been recorded as females,
4) registration of residence 1 patient had erroneously been recorded as a resident in the area in question

Over registration in this material thus amounted to 17 cases (1 7 per cent) due to duplicate registration inclusion of cases with non malignant disease and cases with primarily misdiagnosed site of the primary tumour and, in one case, due to error in recording of residence

Checking of the clinical material with respect to completeness in reporting with the yearly lists of the Registry showed that 17 out of 252 patients submitted to thoracotomy and 9 out of 137 consultation cases had not been recorded in these lists However, a check against the complete lists in 1970 for the years in question revealed that 11 cases had been recorded under category 162 1 4 quite correct 1 as a resident in another area due to incorrectly given address in the report and 6 cases in the special lists of benign tumours Three of these 6 cases had been classified as bronchial adenoma and metastases had appeared in the subsequent course One patient recorded in the benign lists had been reported to the Registry as lung tumour, unspecified whether benign or malignant According to this physician and the death certificate, this patient died later from carcinoma of the lung The remaining 15 patients had also been reported but had been recorded as follows

1) 7 under category 163 (lung cancer, unspecified whether primary or not) In 3 of these cases the reports were defective
2) 2 in other specific sites They had been resected for primary lung carcinoma and also had another primary malignancy but only the non pulmonary site had been recorded,
3) 2 in site 199 (badly defined primary site in thorax) because of vague and inconsistent informations from different sources,
4) 4 in other specific sites (2 on site 164, i e mediastinum) Alteration of the diagnosis after autopsy had not been reported in 2 cases and in the other 2 cases the information of primary site was inconsistent in reports from two sources The diagnosis primary lung carcinoma was secured by re examination

Completeness in reporting was 100 per cent, but 15 (3 9 per cent) of the checked cases had not been listed under category 162 1 and 6 cases (1 5 per cent) had not been classified as cancerous

The Registry data based on yearly lists but reduced with the 17 over registered cases was analysed with regard to basis of diagnosis According to the Registry the diagnosis was based on clinical examination only in 2 cases X ray findings in 21 cytological diagnosis in 24 (2 5 per cent), gross examination at operation or autopsy in 4 and positive histology in 920 (94 7 per cent) Thus the diagnosis was not microscopically confirmed in 51 cases (2 8 per cent) However 19 of these patients had been submitted to post mortem examination and 3 patients to thoracotomy The study of the completeness in reporting (page 391) showed that 26 patients (including those adequately recorded in the complete lists) should be added to the Registry series of primary lung cancer based on the yearly lists On occasion one unreported patient with X ray diagnosis of primary lung cancer was

TABLE 3 *Microscopic Confirmation of the Diagnosis of Cancer of the Lung, Primary and Unspecified Both Sexes, All Ages*

Cancer Registry Time period	Site	Microscopic confirmation (%)
California, USA 1942–1956	162 –163	81 2
Connecticut USA 1964	162 –163	83 2
Denmark 1958–1962	162 1 163	71 8*
Finland 1964 1966	162 163	78
Norway 1959 1964	162 –163	84 3*
South Metropolitan England 1960	162 –163	58 0*
Sweden, 1959–1965	162 –163	94 1
Two districts of Sweden 1959 1965	162 1 163	94 7

* Cytological evidence excluded

Clemmesen 1969) In Finland the registration is based on compulsory reports from hospitals pathological laboratories and private practitioners and listings of all deaths in Finland are annually checked against the files of the Registry Lung cancers reported in death certificates only (Nos 162 and 163) are usually regarded as primary tumours During the period 1964–1966 only 3 9 per cent of all lung cancers (including cases unspecified as primary or secondary ones) were known to the Registry by death certificates only The majority of such cases are likely to fall into the category without microscopic confirmation of the diagnosis It is therefore of interest to refer to Table 3, which shows that in the whole Registry material in Sweden positive histology and/or cytology occurred in 94 1 per cent for site 162 and 163 combined during the period 1959–1965 whereas in Finland the corresponding figure during the period 1964–1966 was 78 per cent It is probable that part of the difference between Sweden and Finland in microscopically confirmed cases is due to inclusion of cases known by death certificates only in the Finnish series but as stated above, such cases amounted to only 3 9 per cent. It should also be pointed out in this context that those cases which are notified by death certification only often are such cases which have never been hospitalized for malignant disease and in most of them the diagnosis has been based on incomplete examination in the terminal stage of the disease The problems of faulty certification have been discussed by several authors, among them *Registrar General*, 1953, 1957, *Gilliam* 1955, *Moriyama et al* 1958, *Case* 1958, *Pedersen & Magnus* 1959, *Willis* 1960, and *Rosenblatt et al* 1969

In the present study the disease was diagnosed less than one month before death or only at autopsy in 30 per cent of the 746 cases not submitted to surgery and 50 per cent of these patients were more than 70 years old

Clinical misdiagnoses are made in a high proportion of cases according to *Willis* 1948, and more recent evidence presented by the *Registrar General* 1957, *Teir & Konttuniemi* 1959, *Garland* 1959, *Barclay & Phillips* 1962, *Rosenblatt et al* 1966, 1969, and *Haupt & Zömisch* 1967 Pathological misdiagnoses are made in not few cases and until the last two decades they were very common (*Willis* 1961) This study, however, does not support these statements and the shown diagnostic error in the present study amounted merely to 0 9 per cent

Over registration due to duplicate registration was found in 0 7 per cent in the Registry material This is an effect of the method to have the personal number as the first basis of filing and the name of the patient as the second One patient had incorrectly been recorded by the Registry as a resident in the two districts Thus in all, over registration reached 1 7 per cent in the Registry series

The error as to site registration in the clinical sample of 389 cases amounts totally to 3 9 per cent Here must be considered that in 1 8 per cent the error constituted of listing the cases in site 163 (lung cancer, unspecified whether primary or not) It is to be expected that a proportion of these cases later would be proved to be primary lung

TABLE 2 Comparison of Histological Types by Classification According to WHO Recommendations and Type Recorded in the Registry The Figures in Brackets Are Percentages based on Row Totals

Histological type	Cancer Registry No	Cancer Registry %	Classification according to WHO recommendations: Epidermoid carcinoma	Anaplastic carcinoma*	Adeno carcinoma	Bronchial adenoma	Others type	No histology
Epidermoid carcinoma	224	41 5	173 (77 2)	28 (12 5)	21 (9 4)	0	2	0
Anaplastic carcinoma	234	43	77 (32 9)	107 (45 7)	39 (16 7)	3	4	4
Adeno carcinoma	66	12	2 (3 0)	7 (10 6)	52 (78 8)	4	1	0
Malignant adenoma	4	1	0	0	0	4	0	0
Other types	5	1	2	0	1	0	2	0
No histology	8	1 5	3	2	1	0	0	2
Total	541	100	257 (47 5)	114 (26 6)	114 (21 1)	11 (2)	9 (1 7)	6 (1 1)

* According to Registry's principles 17 cases with large cell carcinoma and 2 with unclassified type are included in the group of anaplastic carcinoma

single index to indicate the reliability of cancer incidence data (*Doll et al* 1966) Though data from different cancer registries are often not comparable because of known and unknown differences in coverage of the registration, in completeness of the information obtained and in definitions and classification (*Pedersen & Magnus* 1968) an international comparison is given in Table 3 It should be pointed out that the data from the different countries do not cover the same time span It will be seen that the frequency of microscopic confirmation in the unrevised two district series of lung cancer (Int List Nos 162 1 and 163) was very high and similar to the figure for the whole country Nevertheless reporting as judged from this study, seem to be quantitatively very satisfactory though a few cases had been notified after such a delay that they had not been included in the annual reports from the Registry The frequency of microscopic confirmation in the sample used for checking completeness in reporting was equal to that in the Registry series The samples contained a large proportion of patients submitted to surgery (65 per cent) but in the other group (35 per cent) there was a considerable number of patients in whom a primarily ill defined malignant disease of the lung was not found to be a primary lung cancer until during the subsequent course or at autopsy It cannot be claimed that this test sample is definitely representative, but the reporting of clinically diagnosed cases seems to be very satisfactory though probably not one hundred per cent

The significance of the fact that death certificates have not been used by the Registry for registration purposes must be considered The proportions registered as a result of death certification only in different countries cannot be directly compared with one another because the figures do not imply the same thing (*Doll et al* 1966) In Denmark where malignant neoplasms are not notifiable by law 10 per cent of all cases recorded under category 162 1 and 163 according to Int List was known to the Registry by death certificates only during the period 1953 1957 (Source *Clemmesen* 1965) and 6 2 per cent during the period 1958–1962 (Source

volved now and then were reminded of the importance of reporting alteration of a diagnosis Noting in the hospital files, not only of time of reporting but also of notified site, could probably make it easier to detect reported faulty diagnoses The accuracy of diagnosis may be improved by increasingly attention to diagnosis even among older people, and consequent follow up of patients with suspected lung cancer Otherwise the best way of improving the reliability of diagnosis seems to be careful post mortem examinations of as many as possible, especially of those with obscure disease or malignant disese with unknown site of the primary tumour *Berge & Lundberg*, 1970, have recently shown that a considerable part of cases of lung cancer are not diagnosed before autopsy In the mortality series of all cases of lung cancer (74 per cent specified as primary and 26 per cent not specified as primary or secondary) in Sweden 1964–1966, the frequency of autopsies was 63 per cent among males and 65 per cent among females (Source Statistical Reports 1969, Be 1969 3) The errors in site classification and malignancy coding shown in this study stresses the importance of careful coding and the need for inquiries when informations from different sources are insufficient or inconsistent The loss of cases caused by the fact that the Registry has omitted to record cases known by death certificates only is probably small with regard to primary lung cancer The percentage of hospitalized patients with this disease is very high In Denmark 1958–1962 it was 99 7 per cent in the case of primary lung cancer and 93 8 per cent if lung cancer, unspecified as primary or secondary, were included The frequency of clinically diagnosed not hospitalized cases for site 162–164 was only 0 8 per cent in Norway 1959–1964 (*Pedersen & Magnus* 1968) The reliability of histological type registration could be improved if the Registry utilized all available information concerning the histological classification and, no doubt if the pathologists followed generally accepted standardized criteria of typing

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cancer but such an establishment is usually not reported to the Registry. In a few cases incorrect coding was observed. In 6 cases (1.5 per cent) the tumour was coded as benign by the Registry. In 3 of them the tumour had been classified as bronchial adenoma but had later metastasized. On the other hand, it can be seen in Table 2 that 7 out of 541 checked cases recorded as carcinomas in fact were adenomas. The problem how to register bronchial adenomas is difficult because their malignancy is not always shown histologically. It is wellknown that even when definitely malignant, this type of tumour has a very favourable prognosis. Therefore survival rates in cases of lung cancer based on Registry data may be considerably affected by inclusion or non inclusion of such cases. In the present revised Registry series of 998 cases, 214 had been resected and 63 of these patients survived for 5 years or more including 9 out of 10 resected patients with so called bronchial adenoma with or without proven metastases (*Larsson* 1970, unpublished data).

Considerable differences were shown between frequencies of the different histological types as recorded in the Registry and those after re classification by a pathologist using standardized criteria and examining all available histological material. The most important difference was observed in anaplastic carcinoma. The frequency of this type diminished from 43 to 26.6 per cent due to re classification into epidermoid carcinoma and adenocarcinoma. In the yearly reports from the Registry it has been stated that the anaplastic group probably was erroneously over registered and it is of interest that by this study it may be possible to estimate the error. In the case of low differentiated tumours, is is wellknown that squamous or adenomatous differentiation may be difficult to discern and it may be present in only small parts of the tumour tissue so that a difference between a registration of histological type often based on a restricted material at the time of reporting and the final consideration using all available material is likely to occur. The Registry also includes in the anaplastic group cases reported by the pathologist as bronchial carcinoma without further specification and thus an unknown number of actually differentiated tumours are included.

Less explainable is the fact that 22 per cent of epidermoid carcinomas in the Registry files were re classified as anaplastic carcinomas or adenocarcinomas. Here the reporting pathologists must have misjudged the differentiation picture but as mentioned above the reported diagnosis is often made on small biopsy specimens and the tissue often damaged. The pathologists also differ appreciably with regard to experience diagnostic criteria and the extent to which their diagnosis is specified in routine diagnostic work. Although the results of this investigation seem to be rather discouraging when estimating the possibility to make valuable registration of frequencies of histological types one must bear in mind that bronchial carcinoma belongs to the most difficult lesions with respect to histological typing. However when the material was divided into only two histological groups according to Kreyberg the conformity with the re classification was rather satisfactory (84 per cent in group 1 and 86 per cent in group 2). The sample used for this study consisted of patients collected in a large clinic of thoracic surgery and around 60 per cent had been submitted to surgery. It is wellknown that the frequency of epidermoid carcinoma in surgical series is very high and therefore it cannot be claimed that this sample is representative for the whole Swedish lung cancer material.

The errors revealed in this study are insignificant as to registration of sex and residence. The over registration of 0.7 per cent stresses the importance of correct reporting of personal number to the Registry. Over registration due to diagnostic errors and lack of report of altered diagnosis is difficult to avoid but much could probably be gained if every cancerous autopsy was reported and the clinicians and other staff members in

maximum diameters of two different explants were examined microscopically for each point. The viability of the primary explants was estimated and the ratio of viable explants was calculated from the total as a percentage. The grade of growth was recorded as the maximum number of lacunae layers which the growth had penetrated into the supporting matrix. Mean values for the different sera and the different tumour groups on the respective days (3, 7, 11 and 14) were calculated. Because of the apparently uniform growth behaviour of the tumours, as shown in Fig. 1 for all benign and malignant tumours grown in clumping and non-clumping serum, the values obtained on the different termination days were added and the calculated mean values therefore stand for the growth capacity of the tumours within the entire 14-day period of cultivation. The values were analysed statistically by applying Student's t test.

RESULTS

Comparison of the Effect of the Different Sera on the Growth Capacity of the Tumours

All Tumours

All cultured brain tumours were first analysed as one group (Figure 2). The growth

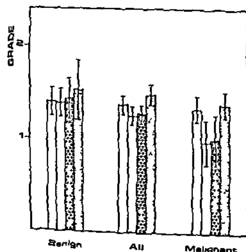


Fig. 2. Mean values with standard error of the means for the penetration into the matrix of all investigated tumours and separately for benign and malignant tumour groups. Columns from left to right indicate explants grown in autologous serum, aged serum pool, clumping serum and non-clumping serum. Note the difference in growth promoting capacity of clumping and non-clumping sera in the malignant tumour group.

in the presence of autologous or non-clumping sera was, under our experimental conditions, slightly better than the growth observed with aged serum pool or clumping sera. No statistically significant conclusions can, however, be drawn. In general the tumour explants penetrated into the first and second layer of lacunae of the matrix.

Clinically Benign and Malignant Tumours

In the group of clinically malignant tumours the autologous and non-clumping sera were significantly better growth promoters than the aged serum pool and clumping sera respectively ($p = 0.05$–0.01). In the group of clinically benign tumours, once again, no differences in the various serum groups could be detected (Fig. 2). Benign and malignant tumours showed practically the same degree of penetration into the matrix whether grown in autologous or non-clumping sera. However, if aged serum pool or clumping sera were used the malignant tumours showed a significantly lower penetration into the matrix than the benign tumours ($p = 0.05$–0.01) (Fig. 2).

Histologically Different Tumour Types

There was almost no serum dependent variation in the tumour growth within the groups of fibromatous meningiomas (group I), meningotheliomatous meningiomas (group II) and neurinomas (group III) (Fig. 3). In the glioma group (group IV) the most abundant growth was recorded for those tumours explants grown in autologous or non-clumping sera, whereas the aged serum pool and clumping sera were less effective or even inhibitory to the growth (Fig. 3). The deviation was statistically significant ($p = 0.05$–0.01).

As previously shown (*Holmström et al.* 1970), the growth capacity of the fibromatous meningiomas (group I) was in the system used significantly higher ($p < 0.01$) than that of the other tumours. This difference was also recorded in the present material and it remained the same in all the sera investigated (Fig. 3).

The best viability was maintained in explants grown in fresh autologous serum In an organ culture technique developed by *Wolff and Wolff* (1961) where chick mesonephros was used as a supporting and nutritive substrate, both maintenance and proliferation of human tumour explants was achieved Long-term organotypic culture of human malignant tumours has been possible in this system (*Wolff* 1963)

The sponge matrix method developed by *Leighton* (1951 and 1954) and *Leighton et al* (1967) also enables the study of both proliferation and maintenance of tumours *in vitro* An essentially similar three-dimensional matrix can be provided by the fibrin foam introduced by *Kalus et al* (1966, 1968a and 1968b) In the latter system not only the original explant structure is retained, but also original histological structures can be reproduced by the growing cells in the matrix lacunae

We have grown human brain tumour explants on fibrin foam in the presence of autologous sera, and shown a good correlation between growth capacity measured on histological sections by estimating the penetration into the matrix, and autoradiographically by tritiated thymidine uptake (*Holmström et al* 1970) Among the histologically different brain tumours, the fibromatous meningiomas showed a significantly better growth potential than the other tumours studied, *i e* meningotheliomatous meningiomas, neurinomas and gliomas The aim of the present study was to investigate whether these brain tumours using an analogous test system, would respond to the growthmodifying capacity of different fresh human sera

MATERIAL AND METHODS

The tumour material consisted of 22 clinically benign and 13 clinically malignant tumours The tumours were subdivided according to their specific histologic type into four groups, which consisted of seven fibromatous meningiomas eight meningotheliomatous meningiomas, seven neurinomas, and ten gliomas graded according to Kernohan to II IV (*Kernohan et al* 1949) The remaining three tumours in the malignant group in addition to the gliomas were two malignant ependymomas and one malignant papilloma of the coroid plexus

Details of the technique used have been described previously (*Holmstrom et al* 1970) Briefly, small pieces of the tumours were cultivated on a matrix of human fibrin foam (Institute of Sera and Vaccines, Prague, Czechoslovakia) in a culture medium containing 90 per cent of Eagle s basal medium (BME (Diploid), Grand Island Biological Co, Chagrin Falls, Ohio, USA) supplemented with 80 IU of penicillin and 40 µg of streptomycin per ml and 10 per cent of the respective serum A known clumping serum a known non clumping serum and the autologous serum were used All sera were fresh and non inactivated, and as a reference, aged and pooled non inactivated random human sera were used since the differences among these sera have been shown to disappear during storage at + 4°C (*Penttinen and Saxen* 1957) The autologous sera were not investigated as to their clumping or non clumping ability However the incidence of these extreme serum types in the population is very low as shown by *Saxén and Penttinen* (1961) Four cultures of each serum type were prepared and the dishes incubated in humidified air and 5 per cent CO_2 at 37°C

After 3, 7, 11 and 14 days of cultivation one dish of each serum type was harvested The cultures were fixed in Carnoy s solution and embedded in paraffin using routine methods Histological sections of the fragments were cut at right angles to the surface, the slides stained with orcein dipped in xylene, and cover glasses were applied with Canada balsam Three sections representing the

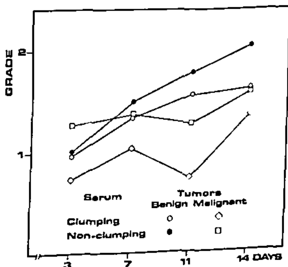


Fig 1 Comparison of growth controlling capacity of clumping and non-clumping sera for the investigated benign and malignant tumours Note the rather uniform growth behaviour of the tumours with no overlapping of the two sera in either tumour group

DISCUSSION

As demonstrated previously (*Holmström et al* 1970) the estimation of growth in our fibrin foam organ culture experiments, by *measuring the degree* of penetration into the lacunae of the supporting spongy matrix, gives reproducible results that correlate well with the proliferative activity of the tissue However, the actual growth is rather limited, and a much deeper penetration into the matrix would be desirable in order to apply the system to the study of growth responses of tumour explants to more subtle environmental, chemotherapeutic or other changes For the present purpose of registering the responses of human brain tumour explants to various types of non inactivated human sera, the system provided sufficient resolving power for demonstration of statistically meaningful differences

As in the previous report, the fibromatous meningiomas *were by far the best growers* No great differences in the general growth capacity of the rest of the tumour groups could be seen When comparing the growth of the tumours divided according to their clinical malignancy, *the* malignant tumour *group demonstrated a significantly poorer* growth in the presence of fresh clumping *serum or non* inactivated aged human serum *pool* This phenomenon could not be seen in the benign tumour group, and closer analysis of the results indicated that the difference was due to the growth responses of tumours belonging to group IV representing malignant gliomas These results are in complete agreement with those presented by *Röller et al* (1966) In their system also autologous *non inactivated sera were found* to be the best protein supplement in the growth medium This does not explain, however, *the good* growth promoting capacity of the non clumping sera, which were as good growth supporters as the autologous sera

The differences in the *effects of fresh* human sera on monolayer cultures have been rather conclusively *shown* to be due to the binding of serum constituents to the growth substrate *of the cells* The treatment of the glass surface with various fresh human sera, prior to plating of the cells on it, *generally* reduced *the attachment of the cells, and the* strongest effect in this respect was caused by the clumping type of sera (*Nordling* 1967) Thus difference could be noted with all tissue culture cell lines studied whether they represented normal *diploid cells or transformed*, established lines Although the serum effect was mostly indirect, the possible presence *of some degree of direct action* on the cells could not be excluded and, in fact, some data, particularly those concerned with the division behaviour of *cells* grown in the various sera, suggested this (*Saksela* 1962) In the present system, *the* growth inhibitory effect of clumping sera and non inactivated aged serum pool could possibly be attributed to the latter type of direct effect, because of its selective nature on the target tissue although definite conclusions cannot be drawn Several possible ways of interaction could be visualized between the highly undulating and richly pseudopoded membrane of glial cells and the lipoprotein-rich fraction of the serum, in which the *differences underlying* the typing of the sera are found (*Saxen and Pentlinen 1961*) *In this context it is* also of some interest to note that the autologous sera of glioma patients, which are known to contain soluble antibodies against glia-specific *surface* antigen, apparently did not *inhibit the* growth of the tumour cells *A clear* cytotoxic effect *can, however be* demonstrated by autologous *lymphocytes on* glioma cells *exposed as a monolayer*, and analysed according *to the amount of* ^{51}Cr released to the *medium from target cells coated* with the *isotope* (*Wahlström et al* 1970)

As previously mentioned, the growth of the *tumour* explants into the matrix is rather *limited* and has to be increased in order to *make* small differences in growth more easily *detectible* The other disadvantage *with this* type of organ culture *system is the* increasing amount of necrosis *appearing in* the cultures with prolonged *cultivation*. This renders long term studies in *this system* impractical,

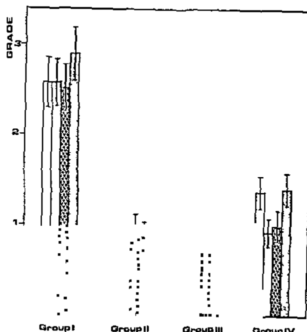


Fig 3 Penetration into the matrix for the histologically different tumour groups, I = fibromatous meningiomas II = meningotheliomatous meningiomas, III – neurinomas and IV = gliomas Mean values with standard error of the means are shown, and columns from left to right indicate explants grown in autologous serum, aged serum pool, clumping sera and non clumping sera Note the significantly higher growth potential of fibromatous meningiomas in all sera used, and the difference of the growth capacity of gliomas in clumping and non clumping sera

Comparison of the Effect of the Different Sera on Viability and Tumour Morphology in the Cultures

In the whole material the over all viability was slightly less than 80 per cent, regardless of the serum supplement in the growth medium The viability decreased gradually from about 85 per cent after three days cultivation to about 70 per cent after 14 days cultivation The various sera used had little effect on the viability (Fig 4) Therefore the values on the different termination days for the sera used were added and mean values for this over-all viability were calculated for the different histological subgroups (Fig 5) In the present tumour material the previously described (*Holmström et al* 1970) higher viability for the fibromatous meningiomas (group I) was also recorded, being about 90 per cent, in contrast to 80 per cent for

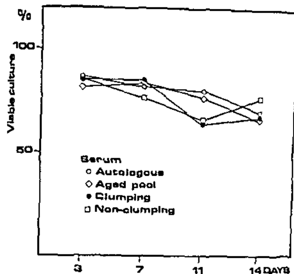


Fig 4 Comparison of the viability on the different termination days for all tumours cultivated The different sera investigated have little influence on the viability

the meningotheliomatous meningiomas (group II) and the neurinomas (group III), and 70 per cent for the gliomas (group IV) (Fig 5)

The tumour morphology of the histologically different tumour types in the present culture system using autologous serum has been recently described in detail (*Holmström et al* 1970) In the present tumour material principally similar behaviour was recorded, and the different sera had no detectible influence on the histological structure and the differentiation *in vitro* of the tumour explants (Fig 6a-d)

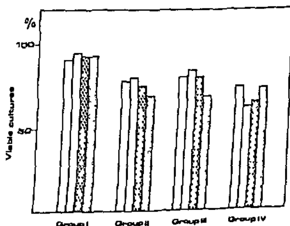


Fig 5 Viability of tumour explants in the histologically different tumour groups *Columns and groups are analogous to those in Figure 3* Note the somewhat higher viability for fibromatous meningiomas

Fig 6 c

Fig 6 d

the tumour is almost the same regardless of the serum supplement in the growth medium The nuclear pleomorphism is abundant in all explants (Orcein stain a 420 × b 240 × c 360 × and d 420 ×)

Fig 6 a

Fig 6 b

Fig 6 Matrix cultures of a astrocytoma gr IV on the 7th day grown a) in autologous serum b) in aged serum pool c) in clumping serum and d) in non-clumping serum The histological appearance of

Acta path microbiol scand Section A 79 407–408 1971

BRIEF REPORT

THYROIDAL C CELLS AND SHORT TERM EXPERIMENTAL FLUOROSIS IN THE RAT

Bengt Sundström

Among the extensive studies and reviews of fluorosis there is still none that has embodied a collateral investigation of the thyroidal C-cells (1, 2) Interactions between fluorides and the parathyroid hormone have been experimentally studied *in vivo* on both ultrastructural and biochemical levels (3) but the possible influence of fluorides on the secretion and blood levels of calcitonin is so far unknown (4)

Material and Methods

As a first approach 16 albino Sprague-Dawley rats weighing 200 g were used 4 rats served as controls 8 rats received distilled drinking water containing 40 ppm F *ad libitum* for 2 months *i e* at a time when a definite fluorosis of the erupting incisor teeth could be seen macroscopically (cf 5) The remaining 4 rats lived together with the controls—drinking distilled water—for 2 months *minus* 4 days at which time they started to receive intraperitoneal injections of an aqueous solution of NaF (20 mg/kg body weight/day in one dose) for 4 consecutive days All three groups contained an equal number of animals of both sexes The animals were killed after ether anaesthesia through neck extension One lateral lobe of each thyroid gland was studied by light microscopy (Bouin fixation paraffin embedding) the other by electron microscopy (double immersion fixation Epon embedding)

Results and Comments

The C-cells appeared morphologically similar in both experiments and they were not different from those of the control animals The sectioned material ... dge ... on of ... —be

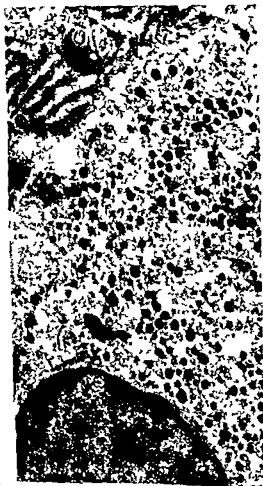


Fig 1 Male rat given 4 acute intraperitoneal injections of NaF (20 mg/kg body weight/day) There was no degranulation of C-cells. × 25 650

Received 30 iv 70 *from Department of Oral Histopathology Odontological Faculty University of Lund S 214 21 Malmo Sweden*

but when these two problems are solved or other assay systems introduced, matrix culture methods could probably be developed for analysis of tumour responses to various external influences, both homeostatic and therapeutic

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COMMENTS ON THE HISTOLOGICAL TYPING OF LUNG TUMOURS

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Director Dr O H Iversen

The demand for more precise clinical follow-up studies, as well as for more differentiated epidemiological surveys, have in recent years increasingly shown the necessity of a more refined morphological differentiation of tumours It is a field for close cooperation between pathologists, clinicians and statisticians and here a common understanding of terms and criteria is essential for a fruitful work This has been realized and resulted in a series of Atlases and Nomenclatures The history of this development shall not be given in the present paper, which will be limited to an analysis of the World Health Organisation Manual Histological Typing of Lung Tumours', published in 1967, through the collaboration of 19 pathologists in 17 different countries

The present analysis is based upon the practical experience obtained by the author through personal use of the manual, as well as acting as consultant to colleagues in a number of different countries

With an international *health* organisation as sponsor of the nomenclature and the definitions their *practical usefulnes* will evidently be the foundation of the analysis

In the preamble of the manual it is said that

The morphological characteristics which define a tumour type microscopically are cytological and architectural, or in daily usage, *histological differentiations*, and this term needs some comment

The normal adult organ is regarded as representing *full differentiation* In tumours, differentiation is more or less impaired qualitatively as well as quantitatively By a *qualitative reduction* in differentiation is meant a diminished manifestation of the cytological and architectural characteristics *of the tissue or organ of origin of the tumour* A *quantitative reduction* in differentiation means an increase in the proportion of tissue showing loss of the specific differentiation of the source tissue In the lung as in other organs, tumours may be described as 'highly , 'moderately' or "slightly" differentiated without justification for any change in typing

If a tumour appears to be without any clearly recognizable component of the source tissue, designations such as "undifferentiated' or "anaplastic" are in common usage In the lung, however, several special tumour types are traditionally recognized in spite of being anaplastic in the sense mentioned Certain cell shapes or certain growth *patterns* are so characteristic that separate types thereby have been defined

The lung is an organ with several multipotent cell types The epithelium lining the bronchi may develop into cylindrical cells that can form glands but, as a result of metaplasia, it may also become highly differen-

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alloted to experimental and control groups Especially, there was neither degranulation, nor signs of intracellular autodigestion of the granules (Fig 1) In fact, if there were any alteration in morphological appearance, this seemed rather to involve the follicular cells, which often showed a distended endoplasmatic reticulum in—both sexes of—the experimental animals This latter impression will be further analysed, sodium fluoride stimulates microsomal adenyl cyclase activity of the thyroid *in vitro* (6), but any such effect following *in vivo* administration appears unknown

The administration of sodium fluoride as a lavage has been found to rapidly, within days, elicit hyperparathyroidism in rats (7) It would follow from the negative results above as regards changes in C cell morphology, that there is no short term release of calcitonin This is in line with the view that the hyperparathyroidism in itself is secondary to a direct (calcitonin *like*!) action of fluoride on bone tissue (cf 8) —Long term experimental studies of the C cells in animals with mild degrees of fluorosis are currently undertaken

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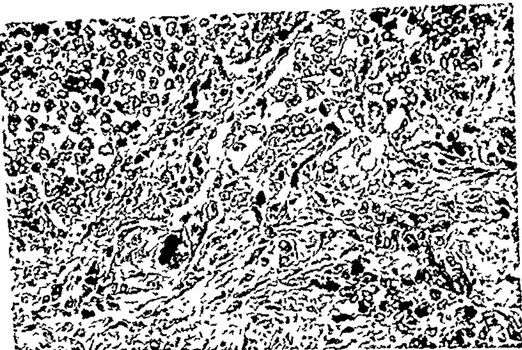


Fig 1 Epidermoid carcinoma + Small cell anaplastic carcinoma (WHO Fig 9)

Delarue indicates illustrations taken from *Les Carcinomes Bronchiques Primitifs* (See References)
WHO refers to illustrations from *Histological Typing of Lung Tumours* (See References)

In the lung however, as just described tumours with little differentiation may be so characteristic as to permit even some of those that are so called anaplastic to be specific ally typed This results in a peculiar dilemma To give an example some parts of a tumour may show a high degree of differentiation characteristic of an epidermoid carcinoma and other parts may consist of a less differ entiated small cell anaplastic carcinoma Such a tumour is presented in Fig 1 (WHO Fig 9) Should the proper type designation here be epidermoid carcinoma partly undifferentiated "small cell carcinoma, partly epidermoid or combined epidermoid and small cell carcinoma ? All three designations may be used by pathologists and each of them might be adequately defended on its own merits In an objective classification, there must be categories for such cases

Fourthly a tumour may be very largely, of a well defined type containing only a very small proportion of cells of another specific differentiation Well known examples are the small cell anaplastic carcinomas, which, as described by *Barnard* (1926) may contain small groups of larger pale cells and small foci of keratinization or vague stratification as well as cylindrical rosettes in the basal layers and abortive gland structures Most pathologists classify such tumours according to the dominant cell type, merely noting the presence of the other cells for future evaluation No doubt, practical considerations based upon previous individual or general experience, including aetiological and clinical information, may be involved in the decisions in typing such cases

Designations such as undifferentiated epidermoid carcinoma should be avoided as self contradictory

Finally, the interpretation of some of the mucin like substances shown by periodic acid Schiff's stain mucicarmine and the alcian stains, and their role in typing is still controversial So far the preamble of the manual

2 *

tiated epidermoid epithelium with marked keratinization

In addition to secretory and duct cells, the normal bronchial glands have been shown to contain myo epithelial cells near the basement membrane and some branched "neuro-secretory" cells recognizable by their granules The latter cells may form tumours with cells of evident epithelial type, which are small, and polygonal and grow in solid masses or trabeculae, sometimes they develop gland like structures and even contain spindle cells

The bronchial glands, like their homologues in other anatomical sites, show production of tumours which, besides conventional epithelium and stroma, may also have components of primitive or mature cartilage like tissue or mucoid masses with stellate cells of epithelial or mesenchymal origin

For lung tumours it has therefore been found rational to establish a scale of diminishing differentiation as follows

(i) Tumours with recognizable components of source tissue (adenocarcinomas, epidermoid carcinomas)

(ii) Tumours with a characteristic regularity of arrangement or cytological features not similarly represented in the source tissue (carcinoids)

(iii) Tumours with mucin like substances, in the absence of clear evidence af gland or gland-like structures

(iv) Tumours that can be recognized only on the basis of the size and form of the cells or their growth pattern, such as garlands, streams, rosettes (small cell anaplastic carcinomas, with subtypes)

(v) Tumours that are completely irregular as to cellular form and size as well as to histological architecture, thereby precluding a specific classification

Tumours of categories iv and v are considered truly "anaplastic" only if they consist of primitive cells

This scale is based upon present techniques and knowledge Advances in histochemistry and electron microscopy will undoubtedly make it possible to recognize other special features and thus further diminish the proportion of tumours that have to be placed in the last category, or even necessitate changes in the present classification

"*Pleomorphism*" is often manifest, especially in anaplastic tumours but does not in itself denote anaplasia, this is exemplified by the phaeochromocytomas where pleomorphism is a constant feature

In anaplastic tumours rapid growth is often evidenced by the presence of necrotic foci and large numbers of mitoses The latter, however, do not necessarily indicate rapid growth, as a large number of mitoses may also be an expression of a prolonged mitotic time (*Iversen 1964*)

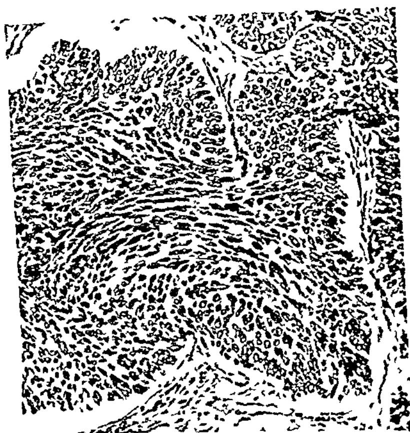
Difficulty in type designations may be caused by the presence in different parts of the same tumour of various degree of differentiation or even of several kinds of differentiation

Firstly, it may happen that two well-defined tumours that begin independently subsequently coalesced, resulting in a true combined tumour, actually these are "collision" tumours

Secondly, features of two well defined types of differentiation originating from the same source cell may be found in the same tumour, for instance simultaneous manifestation of the features of an adenocarcinoma and an epidermoid carcinoma Here, too the designation *combined tumour* is commonly used and acceptable The designation "mixed" should be reserved for other tumours to be discussed later

Thirdly, a lung carcinoma may show some parts that are well differentiated (category i) and others that are less differentiated (category iv) According to common usage in classifying tumours of other organs this should lead to a *type designation based on the most highly differentiated part*, qualified by adjectives characterising the degree of differentiation of less differentiated portions —in other words, a *histological grading which is a process completely separate from "typing"*

Fig 3 Carcinome epidermoide peu différencie Les cellules ont tendance à prendre par places un aspect fusiform (Delarue Fig 18)



responsibility for the result I feel never theless that it is my duty as head of the centre now to present *some of the difficulties encountered as well as to discuss possible future revisions*

WHO Code No I *Epidermoid carcinomas* are defined as tumours with keratinization or intercellular bridges This is a precise but also a very narrow delimitation Stratification and whorl formation are not mentioned whereby tumours with a transitional cell epithelium are excluded Many will find this regretable and if the designation squamous cell carcinoma of the old tradition had been used a field of controversy would have been removed The dilemma is demonstrated by two illustrations

Fig 2 shows a tumour from the WHO manual (WHO Fig 10) where the coding in given as I or II 1 thereby informing the reader about our difficulties Under the circumstances I perfer the Code no II 1 as there are no bridges or keratinization but there is, however, some stratification and a cylindrical basal cell layer

Delarue of Paris (1967) a member of the WHO collaboration centres for lung tumours presents the following illustration Fig 3 (*Delarue* Fig 18) under the designation Carcinome epidermoide peu differencie ignoring the WHO definition (on the assumption that the specific criteria have not been satisfied in other parts of the same tumour)

Delarue circumvents the WHO definitions simply by stating that his own classification is voisine to that of WHO and at the same time in addition creating his own formes particulieres such as epidermoid carcinomas a cellules fusiformes and à cellules claires

The first of these special formes the spindle celled Fig 4 (*Delarue* Fig 20) cor

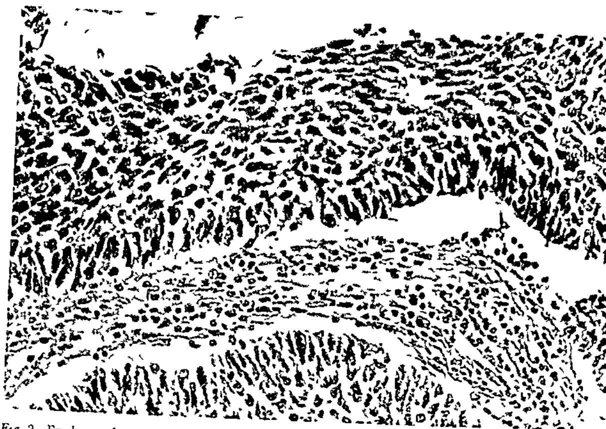


Fig 2 Epidermoid carcinoma or *Small cell* anaplastic carcinoma fusiform cell type? (WHO, Fig 10)

Either agreeing, or disagreeing with the designations or definitions of the WHO pathologists is of minor importance, if only the code numbers are adhered to Then everyone may cultivate their own personal idiosyncrasies, and work towards a revision—for revisions will certainly come The main purpose is to define a sufficient number of types according to objective criteria in order to create a means of common classification

If the stipulated criteria are strictly adhered to, the type seems to remain the same throughout the lifetime of the tumour In a combined tumour it may, however, happen that one of the components is gradually outgrown and disappears Such an example can be seen in tar cancer in mice, where the original tumour shows an epidermoid and a fusiform cell component, but eventually the fusiform cells prevail Such cases, however, seem to be extremely rare in human material Quite another thing is that a tumour during its growth and spread can manifest an increasing or decreasing *degree of differentiation* The soil may be of importance in this connection Most pathologists are familar with the fact that lung tumours in brain metastases show an especially marked differentiation, sometimes also in other metastases for instance in lymph nodes

The main points in every classification are (i) to have as mentioned a sufficient number of "pigeon holes' (*i e* of code numbers) and (ii) to have a consistent philosophy The second is not as easy as the first A consistent philosophy may be difficult enough for anybody, but it is close to impossible for a large committee, consisting of members drawn from different countries with different traditions A chairman with strong convictions and a strong will, trying to impose his views, may provoke considerable resentment among the other committee members One the other hand, a weak chairman may bring his committee to imprecise conclusions

As regards lung cancer classification, the subject of the present paper, I have to state that, I accepted compromise decisions In, however, accepting my full share of the

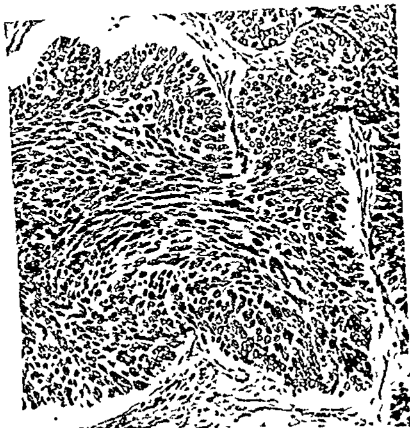


Fig 3 Carcinome épidermoïde peu différencié Les cellules ont tendance à prendre par places un aspect fusiform. (Delarue, Fig 18)

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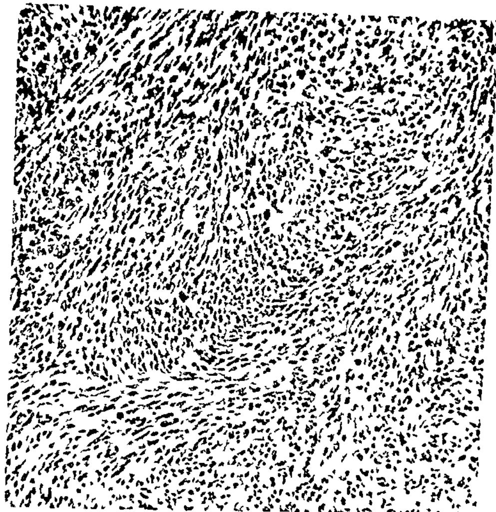


Fig 4 Carcinome epidermoide à cellules fusiformes (Delarue, Fig 20)

responds to the WHO Code No II, 1, as shown in WHO Fig 3, here Fig 5

The second one, as 'cellules a manteau", Fig 6 (*Delarue* Fig 13), resembles the WHO Code No IV 4, large cell carcinoma, here Fig 7 which will be discussed later

When *Delarue* states that he uses a classification "voisine' to that of WHO, and at the same time introduces formes particulieres" I can easily understand his viewpoint and even sympathize with the deeper meaning of his attitude, because behind his viewpoint there seems to be an undercurrent of biological recognition, with which I agree The trouble is that our mandate was to propose a histological typing, and we were accordingly primarily *bound to morphological criteria* This essential dilemma will be constantly encountered in the present review, actually at the next code number

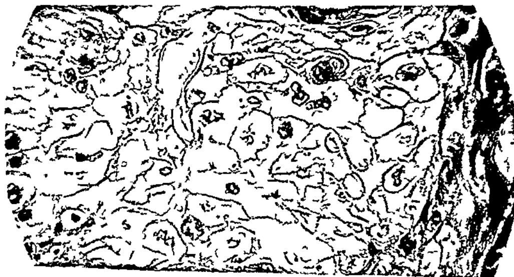
WHO Code No II, *Small Cell Anaplastic Carcinomas,* are defined as 'highly malignant tumours that metastasize early and widely, by both the lympatics and the blood vessels They comprise a number of morphological sub-types

1 *Fusiform cell type*
2 *Polygonal cell type*
3 Lymphocyte like (oat cell') type
4 Others

As already discussed, a number of pathologists tend to classify sub types 1 and 2 as 'undifferentiated epidermoid carcinomas , a truly self contradictory term However, that is the reason for placing them close to the epidermoid carcinomas

For sub type 3 the designation oat cell has been retained as a synonym against considerable opposition in order to conform with the whishes of many pathologists and clinicians

F g 5 Small cell anaplas c carc noma fusiform cell type (WHO F g 3)



F g 6 Carc nome ép dermo de. Cellules à manteau (Delarue F g 13)

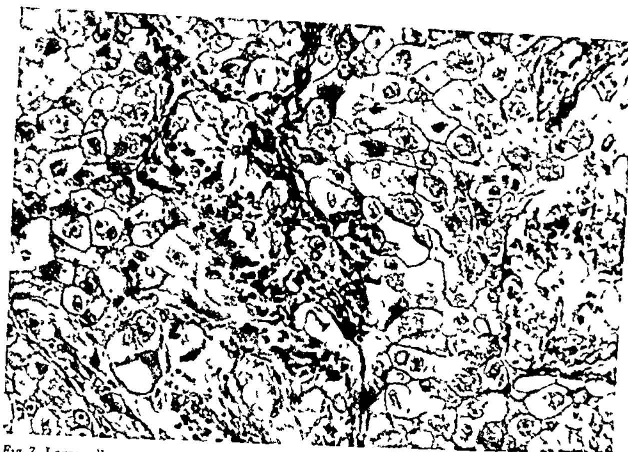


Fig 7 Large cell carcinoma, solid type, with mucin like content code IV, 1 (WHO *Fig* 15)

Sub-type 4 is reserved for rare forms with rosettes, tubules and other structures

There seems to be general agreement that Code No II is not an entity, and the main reason for *ad interim* still maintaining these tumours as one type is purely practical In the ordinary *routine examination* of material presented for diagnosis, it may be impossible to distinguish between the different sub types The slang diagnosis is often simply oat cell and that means for many surgeons abstention from radical operation The sub tleties of a more *refined typing* is a later step for the pathologist in collaboration with the clinician The connecting bond in the WHO classification is actually the high malignancy of all these sub types, that is a *clinical criterion* This explains the devia tion from the surely morphological principle in the definition of this main type In the future, techniques better than those used now in the ordinary laboratories, such as enzyme histochemistry and electron microscopy will add to our knowledge and give a better basis for a more rational typing A beginning has already been made, as will be mentioned later

WHO Code No III, *Adenocarcinomas* are tumours whose basic morphological char acteristic is the 'formation of tubules and gland like structures , sometimes combined with papillary growth and/or mucin forma tion

The main difficulties regarding this type are connnected with, 1) the evalution of what is to be accepted as tubules and gland like structures and 2) the evaluation of the presence of tiny amounts of mucin like sub-stances Sometimes the problem is actually solved by the simultaneous presence of both of these features even if each is found only *in traces*

The separation of sub type III, 2 is again based partly upon morphological and partly upon clinical evidence, as given in the WHO manual

WHO Code No IV, *Large Cell Carcino mas*, are defined as tumours composed of

large cells without evidence of epidermization or formation of gland like structures As a rule these behave clinically much like bronchogenic adenocarcinomas Again sub types are distinguished

1 Solid tumours with mucin like content
2 Solid tumours without mucin like content
3 Giant cell carcinomas
4 'Clear cell' carcinomas

It is obvious that this main type is not an entity, and several problems are involved

Sub type IV,1 is characterized by solid heaps of cells, some of which show mucin reaction to the proper stains If such reaction is totally missing, the tumour belongs to sub-type IV, 2, or sub type IV, 4, if the cyto plasm is "clear' *To distinguish between sub-types IV, 1, IV, 2 and IV, 4, staining for mucin is therefore necessary* It should be mentioned that degenerating nuclei sometimes take the mucin stains, thereby mimicking a mucin positive tumour

Sub type IV, 4 especially shows many features resembling the tumours of 'cellules a manteau' of the French school, some of the tumours possibly biologically related to the epidermoid carcinomas

An important problem here is the interpretation of substances which stain with one or all of PAS, mucicarmine, the alcian stains and several others Without entering into the complicated problem of definition of mucin, there cannot be any doubt that through the use of such stains important information is obtained Among other things, a number of slightly differentiated adenocarcinomas can be identified, which would be missed in ordinary routine H E stained slides On the other hand certainly other lung carcinomas do occur, having an intracytoplasmic accu mulation of material which takes the above-mentioned stains, but without showing any structural features permitting a diagnosis of adenocarcinoma It has never been claimed from our laboratory that the mere presence of such mucin like substances can alone justify a diagnosis of adenocarcinoma To this must be added a technical difficulty We have seen tumours which shortly after fixation and embedding contained mucin positive cells, but after a couple of years, sections cut from the same block were negative in their response to such stains Without having made systematic studies, we think that fixation in an acid formalin (un neutralized, or with acetic acid added) preserves the mucin like substances better than neutral formalin

Secondly is the question of the solid heaps of undifferentiated tumour cells (Sub-type IV, 2), the "carcinoma solidum' of former days If such cells are found in an adenocarcinoma, a number of phathologists tend to diagnose "combined epidermoid adenocarcinoma", ignoring the criteria stipulated for epidermoid carcinomas these tumours even lacking stratification Undifferentiated epidermoid carcinoma is, according to our criteria, not an admissible diagnosis, as already mentioned

Sub type IV, 3, Giant cell carcinomas, ought for systematic reasons to have been No 4 It is a question if these are not sometimes biologically epidermoid and more often adenocarcinomas, highly anaplastic and pleomorphic, with some of the cells grossly distorted as a result of degenerative changes

This main type, the large cell carcinomas, has in recent years been considerably reduced in number, because more and more of the tumours have been identified and properly placed as other types, especially adenocarcinomas

WHO Code No V, *Combined epidermoid and Adenocarcinomas* are stated to be 'comparatively rare tumours which should in some parts have definite evidence of epidermization as well as features that would qualify other parts unequivocally as adenocarcinoma" It is important to stress the words "definite' and "unequivocally" It should be remembered that an epidermoid carcinoma infiltrating and engulfing bronchial glands may lead to erroneous conclu sions, as does the finding of single layers of basal cells in an autolytic epidermoid car-

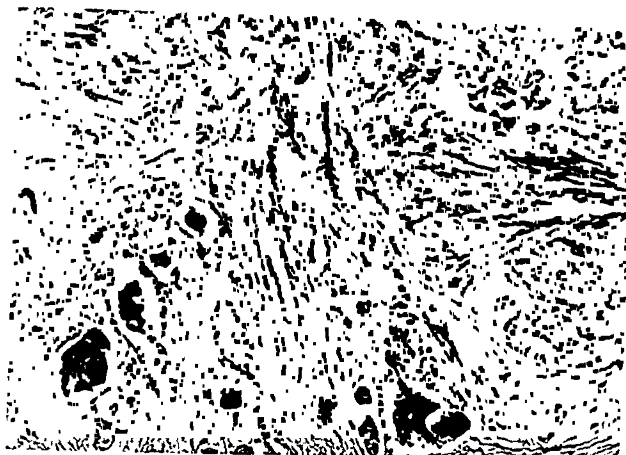


Fig 8 Epidermoid carcinoma, operation specimen The tumour had been treated with anti mitotic drugs The degenerative *changes may* mimic a "combined epidermoid adenocarcinoma"

cinoma with degenerating cells in the middle of a pseudo-lumen also *mimicking an adeno*carcinoma Such cases are often found in epidermoid lung carcinomas treated by antimitotic drugs or by irradiation, as shown in Fig 8 True combined tumours should exhibit two types of differentiation from the same source cells, here the cylindrical epithelium, lining the bronchi

WHO Code No VI, *Carcinoid tumours* "Most of these are centrally localized and usually show a mosaic or trabecular arrangement of polygonal cells with somewhat granular cytoplasm and regular oval nuclei They may have an acinar structure or be composed of spindle-shaped cells, especially when they originate in subsegmental bronchi Some of these tumours contain argyrophilic granules which correspond to the "neurosecretory" granules seen under the electron microscope"

The description shows that differential diagnostic difficulties may aries vis-a-vis certain polygonal-type small cell anaplastic carcinomas, as well as those designated as composed of "lymphocyte-like" cells ("oat" cells) The situation is further complicated by recent findings of "neurosecretory" granules with clinical *effects* also in "oat-cell" carcinoma of the lung, leading to the suggestion "that oat-cell pulmonary cancer and bronchial carcinoid tumours are closely related" (*Bensch et al* 1968) What "related" means will be discussed later

WHO Code Nos VII, *Tumours of Bronchial Glands* and VIII, *Papillary tumours of the Surface Epithelium* do not seem to raise many problems

WHO Code No IX, *"Mixed Tumours and Carcinosarcomas,* on the other hand offer some problems of definition and of interpretation

Sub-type IX, 1, *'Mixed tumours"*, comprises "tumours with gland-like structures closely associated with masses of stellate cells

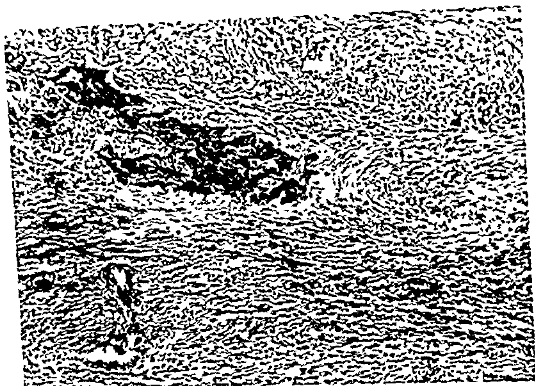


Fig 9 Carcinosarcoma, same case as illustrated in WHO Fig 32

similar to those of cylindromas, and sometimes also with masses of cartilage like tissue These tumours often greatly resemble certain tumours of the salivary glands and their *homologues* (the lachrimal glands) to which the bronchial glands probably belong

The stellate cells in their mucoid ground substance as well as the cartilage like tissue greatly resemble mesenchymal differentiation That is the background for the old traditional designation of 'mixed tumours Today most students are inclined to regard these mesenchyma like differentiations as of epithelial origin (myo-epithelial cells, neuro secretory cells) This would mean that even if the mixed tumours should properly be *regarded* as truly epithelial the adenomatous and mesenchyma like components might stem from *different source cells*

Others of the WHO centres are on the point of adopting the term pleomorphic adenomas for these tumours, to me an unfortunate decision.

Firstly, 'adenoma' is, according to common morphological and clinical definitions a benign tumour All medical students are taught this, and it cannot be more simply stated than as follows 'An adenoma is an *innocent* epithelial tumour of glandular structure, etc' (*Boyd* 1961) However, these mixed' tumours are not at all innocent' They are recurring in a high percentage for two reasons i) tumour tissue is often found between the lamellae of the capsule surrounding the tumour, resulting in an incomplete removal, if a fairly radical resection is not carried out ii) dissemination of tumour cells easily takes place if the tumour capsule 'bursts' during removal Under such circumstances implanted tumour cells have a great tendency to grow into tissue interstices and show a strong tenacity , as I used to express this cellular property to the students

Philological considerations should not override these very important clinical facts, and besides that the designation adenoma is not

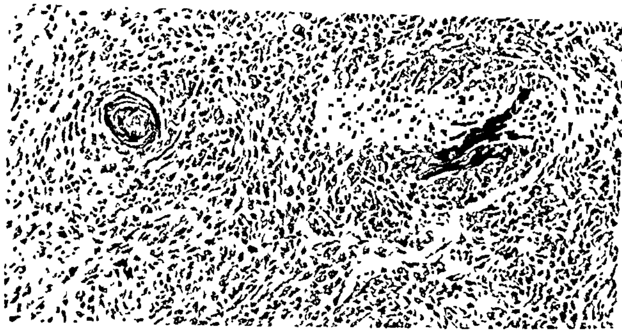


Fig 10 Carcinome epidermoïde Association de cellules fusiformes et de formations keratinisées (Delarue, *Fig 21*)

covered by common usage in such tumours "Semi-malignant" has likewise been rejected by the *same centres as a clinical* characterisation, in spite of the fact that these tumours are neither malignant in the true sense, nor clinically being The pathologists, the clinicians, and more important still, the patients should be spared the disastrous results of a designation which indicates that these tumours are unreservedly benign adenomas

The sub-type IX, 3, *Other carcinosarcomas* may also be subject to differences of opinion The tumour reproduced in the WHO manual (as Fig 35), here Fig 9 was in my own files described as an "epidermoid tumour with fusiform cells" A very similar tumour is designated by *Delarue* as 'Carcinome épidermoïde Association de cellules fusiformes et de formations keratinisées" (Delarue Fig 21), here Fig 10 *Drury and Stirland* (1959), on the other hand, use the term *"carcinosarcomatous'* for a very similar tumour The adjective form marks a subtle distinction

The designation should be determined by the true character of the more or less polymorphous spindle cells If they are of mesenchymal origin, a sarcomatous component is present If the cells are very anaplastic epithelial cells, then the designation sarcoma lacks foundation The *question is, however,* of small practical importance, even if of some academic interest The main point is to give these tumours a code number (which can be as anyone pleases *for his own special purposes*)

Practical histological typing of tumours is usually performed on fragments of tissue, taken at a *certain stage only*, of a long development From this several problems arise

(i) Is the type the same in all parts of a tumour?

(ii) Is the type constant during the lifetime of a tumour?

(iii) Does the histological type give any biological information?

(i) The first question has already been touched several times It has been stressed that "combined' and 'mixed' tumours are fairly rare and make up only a small percentage of *all*, and the two components are usually so closely mingled that even in ordinary routine material the observation of the two components is noted When, in spite of this some pathologists still claim that several types often are found represented *in the same* tumour, the explanation most probably is

that the criteria, as stipulated in the WHO manual, are not strictly observed The errors mentioned earlier are fairly common When the WHO team wanted a good illustration of a combined epidermoid adenocarcinoma for the manual, it took a long time to obtain a satisfactory sample At a recent seminar in Oslo (*December, 1968*), held by *Scandi-* [illegible] I took great pains in [illegible]

examples were found, along with quite a number of spurious ones Furthermore, the epidemiological data, especially the response to tobacco smoking shows such a marked distinction between epidermoid and adenocarcinomas that a great number of combined types may for such reasons be excluded (*Kreyberg* 1969)

(ii) The second question is probably related to the first Possibly most pathologists will agree that with the clinical development of a spread of malignant disease (or metastases), the same tumour type is encountered at all sites, possibly with different *degrees* of differentiation (lung, lymph nodes, brain), as already mentioned

(iii) The answer to the third question is partly indicated by the fact that some clinical (biological) characteristics have been used *in the very definition itself of some of the* types For instance, the high malignancy with early metastases of the Type II tumours, and the occurrence of neurosecretory granules, accompanied by hormonal symptoms in some of the carcinoids (Type VI) and small cell anaplastic tumours (Sub type II 3) Clinical considerations are also involved in the separation of the bronchiolo-alveolar carcinomas (Sub type III, 2), from the conventional adenocarcinomas (III 1)

It is now increasingly accepted that the Types I and II are the main tumours of the lung provoked by exposure *to uranium ore*, nickel chromium tar fumes and tobacco smoking This was first revealed through the increasing *sex ratio* of these types for which reason they were grouped together under the designation Group I tumours (*Kreyberg*), in contrast to all the others (except Types IV and V), *called Group II* The *ratio Group I Group II* is actually expressing the same condition in a better way, because with decreasing smoking in males and increasing smoking in females, the sex ratio may change The tumour types are connected with the exposure to the provoking agents and not with the sex as such

Having introduced a certain 'grouping' of types as a special analytical means for a special purpose, this opens the way to other groupings" of the basic types to suit other purposes Prognostically, for instance, the types *II*, III and IV are more related As regards hormonal secretion the Types II (especially Sub-type II, 3) and VI are *related*

It should be remembered that for many purposes it may be more useful to sub-divide the material rather extensively rather than to try to unite entities on the basis of a common denominator in spite of obvious differences

If electron microscopy and histochemical studies some day should reveal that Subtypes II, 1 and II, 2 are closely related to the epidermoid carcinomas (Type I), then a basis for a revision may have been established, but other biological differences, for instance, expressed as differences in malignancy, are still present The nature of the tumours has not changed, only our attitude toward their classification *(grading)*

The aim of the present paper has been to show some of the principles in the WHO histological classification of lung tumours, as they appeared in 1967 The classification was created to give an international tool for research No member of the committee ever believed that the classification presented would be final When asked by one of the highest officers of the WHO in 1967 how long I thought the classification would last I answered 'I hope some 10 years' 'Oh", was the reply, 'then we should be very satisfied This paper is a contribution to the discussion of a future revision of the histological typing of lung tumours

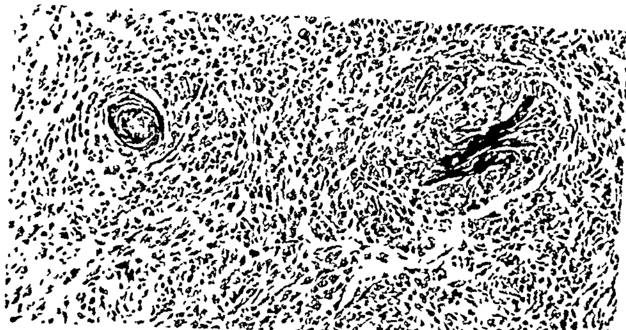


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INTERACTION OF TRANSPLANTS OF THE EHRLICH CARCINOMA

Lack of Local Reaction to Subcutaneous Transplants in the Presence of Late Intraperitoneal Tumour

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Mice with late intraperitoneal transplants of the Ehrlich carcinoma fail to produce an acute inflammatory response to a subsequent subcutaneous transplant of these cells. It is suggested, on the basis of experiments in mice treated with aggregated gamma globulin, and with zymosan, that lack of complement may be responsible for this failure Complement is therefore implicated as a mediator of the acute inflammatory response to these tumour cells, *without which infiltrative tumour cell growth does not occur It was also shown that the* acute inflammatory response to the subcutaneous injection of turpentine may be divisible into two phases, an early complement dependent stage and a later complement independent one

In normal mice a subcutaneous transplant of the Ehrlich carcinoma elicits an acute inflammatory response (*Hartveit* 1969) In mice with a late intraperitoneal transplant a further subcutaneous transplant fails to do so This observation, which is confirmed in the present work, prompted a series of experiments that attempt to find out why the acute inflammatory response should fail in these mice

It is possible that the mice with late intraperitoneal transplants might be unable to produce an acute inflammatory response, due, among other factors, to peripheral circulatory failure This was investigated by testing their response to the subcutaneous injection of turpentine

Further, mice with late intraperitoneal transplants of this tumour are known to have low serum complement levels (*Hartveit* 1964) It was therefore possible that lack of complement might be responsible for the lack of reaction, as complement is a known mediator of the acute inflammatory response (*Willoughby et al* 1969) To test this hypothesis the reaction of complement depleted mice to subcutaneous tumour injection was investigated, and an attempt was made to recomplement mice with late intraperitoneal transplants

Finally the reaction of normal mice and mice treated with zymosan, that is used to inactivate complement factor 3 in vitro (*Kabat & Meyer* 1961), was compared, as it is also said to be active in vivo (*Geerlinger & Sörensen* 1970)

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IIa) *The tumour cells lay in a compact mass separated from each other by the erythrocytes that were injected with them* (Plate IIb)

The findings in all the mice investigated were similar There was no marked difference in the findings at 24 and 48 hrs, and no sex difference was apparent

In contrast the transplants in the controls were pale in colour and surrounded by an area of marked hyperaemia (Plate I b) (The erythrocytes in the tumour ascites appear to float away to the periphery of the transplant in the oedema that forms around it) At 48 hrs the edge of the transplants merged with the host's connective tissues, showing early infiltrative growth (Plate II c), and there was massive tumour cell necrosis at the centre of the transplants The tumour cells were interspersed with groups of granulocytes (Plate II d)

The findings were similar in all the mice investigated, there being a slightly greater inflammatory reaction in the males The transplants at 48 hours were more hyperaemic than those at 24 hours

Experiment 2 Four hours after turpentine injection the 2 female mice with intraperitoneal tumour showed no macroscopical response at the injection site In the male there was slight hyperaemia Microscopically no inflammation was seen in these females although muscle necrosis was present (as judged from loss of striation) and degranulated mast cells were present in the injection area The blood vessels by the injection site showed slight hyperaemia in the male

In the control mice there was hyperaemia and oedema with slight carbon marking of the vessels over an area of approx 0 7 mm Microscopically there was a border of macrophages and granulocytes around the injection site The small blood vessels were dilated and showed margination of granulocytes

At 14 hours all the controls produced a marked inflammatory response with black oedema over an area of about 1 cm² surrounded by diffuse hyperaemia and vessel labelling Both the males in the experimental group showed a slight inflammatory response, with little oedema on macroscopic examination and only slight carbon labelling The females produced an area of oedema similar to the controls, but with less hyperaemia round it These females had smaller intraperitoneal tumours than the males

Histology confirmed that the inflammatory response was less in the males in the experimental group There was no oedema or inflammatory cell exudate in one, very little in the other, although the small vessels were obviously damaged and contained carbon

Experiment 3 At 24 hrs after tumour injection in all the controls there was an area of oedema along the needle track and over the tumour transplant with marked hyperaemia of the vessels leading to this area By contrast the vessels of the abdominal skin were almost empty In the mice treated with aggregated gamma globulin there was much less oedema The vessels showed irregular blood filling that was also evident in the abdominal skin The difference in the reaction between the two groups was as marked at 48 hrs

Plate II e shows the edge of the tumour transplant and the area between it and the panniculus carnosus in a mouse given 0.5 ml aggregated gamma globulin, twice There is little oedema or host cell infiltration in this area compared with that in Plate II f, which is from a mouse given 0 25 ml of the same suspension, also twice These doses are greatly in excess of those used by *Willoughby et al* (1969) to deplete rats of complement

Experiment 4 After 14 hrs there was little oedema and only slight carbon labelling at the turpentine injection site in the mice treated with zymosan (Plate I d), compared to that in the controls (Plate I c) These findings were confirmed histologically Similarly 14 hrs after the injection of tumour cells there was a marked reduction in the inflammatory reaction following zymosan treatment in all the mice (Plate I f), compared to the controls (Plate I e) This was also confirmed histologically

When both turpentine and tumour injections were given to the same mouse the

MATERIAL AND METHODS

Mice of the closed colony kept at this Institute that originated from a group of previously inbred white mice obtained from Professor *Kreyberg* (Oslo) in 1959, were used when between 2½ and 3 months old

The *tumour* was the Ehrlich carcinoma kept by serial intraperitoneal transplantation in these mice at approximately 10 day intervals For subcutaneous transplantation 0 05 ml of the whole tumour ascites from a 10 or 11 day transplant was used while 0 1 ml was used for intraperitoneal transplantation

Turpentine (undiluted) was given subcutaneously in a single dose of 0 01 ml per mouse

Zymosan (Sigma), from *S cereviceae* yeast, was given at a dosage of 20 mg/Kg

Human gamma globulin (Kabi) 8 or 4 per cent solution (w/v) in physiological saline was aggregated by heating to 63°C for 10 minutes (*Thunold et al* 1970) The resultant suspension was injected intravenously, in various doses

Colloidal carbon, Pelican' ink (C11/1431 a) was used as a 1 5 suspension in physiological saline with 1 per cent gelatine added as a stabiliser This was used for intraveneous injection to outline inflamed vessels at a dosage of 0 2 ml/mouse given 1 hour before the animals were killed

Experimental procedure

Experiment 1 Four male and 4 female control mice and 8 male and 5 female mice with 10 day intraperitoneal tumour transplants were each given a subcutaneous injection of tumour cells on the back Twenty four hours later 3 males and 1 female with intraperitoneal tumour were killed, together with 2 male and 2 female controls At 48 hours the surviving mice (2 males and 1 female with intraperitoneal tumour, and 2 male and 2 female controls) were killed

The skin of the back with the subcutaneous tumour was removed This material was fixed in formalin and paraffin embedded as described previously (*Hartveit* & *Halleraker* 1970) Histological sections were stained with haematoxylin and eosin

Experiment 2 Three male and 4 female mice with 10 day intraperitoneal tumour transplants were each given a subcutaneous injection of turpentine on the back Three untreated males and 2 females were given similar injections

One male and 2 females in the experimental group, and 1 male and 1 female control were killed 4 hrs later after carbon injection By this time the carbon had cleared from their general circulation The injection sites were treated as the subcutaneous tumours in exp 1

The remaining mice were treated in a similar way and killed at 14 hours

TABLE 1 *The Volume of 4 per cent Aggregated Gamma Globulin Given in the Experimental Animals Related to the Time of Treatment (See Exp 3)*

No of mice	globulin (ml)	time of injection (hrs)
4	0 5	0*
		24
4	0 25	0
		24
4	0 5	0
4	0 25	0

* at start of experiment

Experiment 3 Six male mice were given 0 5 ml of 8 per cent aggregated gamma globulin suspension intravenously Directly afterwards they, and 4 untreated male controls were given a subcutaneous injection of tumour cells on the back Half the mice in each group were killed 24 hrs later the rest after 48 hrs Histological preparations were made as above

The experiment was repeated using graded doses of aggregated globulin as shown in Table 1 All the treated mice, and an equal number of untreated controls, were killed 48 hours after tumour injection Histological specimens were prepared as above

Experiment 4 Sixteen male mice were used Eight were given 2 doses of zymosan at a 4 hr interval The others were left untreated Four treated mice and 4 controls were then given subcutaneous tumour, and the others subcutaneous turpentine

All were killed 14 hrs later after intravenous carbon treatment Histological preparations were made as above

The experiment was repeated giving both the subcutaneous tumour and the turpentine injections to the same mouse The two injections were kept well apart, the tumour being given high up on the back the turpentine at the tail end A further group was added given intraperitoneal zymosan

Half the mice were killed after 14 hrs the remainder after 38 hrs Histological preparations were made as before

RESULTS

Experiment 1 In mice with intraperitoneal tumour no inflammatory reaction occurred around the subsequent subcutaneous transplant (Plate Ia) The edge of the transplant was well demarcated from the surrounding tissues that lacked host cell infiltration (Plate

response to turpentine at 14 hrs was in each case more intense than that to tumour, and carbon labelling was greater with the former (*Plate I g*) Following intravenous zymosan the response to both turpentine and tumour was reduced and carbon labelling was absent, even from the turpentine injection site (Plate I h) The findings with intraperitoneal zymosan were similar

Histologically the reaction to turpentine was greatly reduced following intravenous

(Plate II g) There was little inflammatory cell infiltration although muscle cell necrosis was present The changes were less marked following intraperitoneal zymosan.

At the edge of the tumour transplant in mice given intravenous zymosan there was a marked lack of inflammatory cells and no carbon labelling (Plate II j), in contrast to the controls (Plate II i) In those given intra peritoneal zymosan a thin band of exudate was present on the surface of the transplant and appeared to confine the tumour cells

At 38 hrs a brisk inflammatory reaction was present in the controls at both injection sites (Plate I i) Following intravenous zymosan the reaction around the tumours was reduced, but not that at the turpentine injec tion sites (Plate I j) The findings following intraperitoneal zymosan were similar

Histology of the tumour transplants did not show any principle differences from the reaction at 14 hrs The development of an acute inflammatory response at the sites of the turpentine injections was confirmed

With these early tumour lesions it was not possible to differentiate macroscopically with any degree of certainty between the edge of the tumour transplant and its surrounding oedema The measurements of the total areas involved (sum of two greatest diameters at right angles, ± S D) are shown in Table 2 The size of the lesion at 14 hrs is repro ducible, and it does not increase in area by 38 hrs, though the standard deviation shows it is less constant Following intravenous zymosan there was a marked and consistent

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Plate I Magnification of the macroscopic pictures is about × 3 in a and b, × 2 in c and d and × 1 5 in the others

a Subcutaneous tumour transplant at 24 hours in mouse with an 11 day intraperitoneal trans plant in addition
Note sharp edge of transplant and lack of hyperaemia in skin vessels

b Subcutaneous tumour in mouse without other tumour (24 hrs)
Note diffuse edge and hyperaemia of skin ves sels

c Reaction of untreated mouse to subcutaneous turpentine injection (14 hrs)
Note marked oedema and carbon labelling (→)

d Reaction to turpentine at 14 hours in mouse treated with zymosan
Note little oedema and only slight carbon la belling

e Reaction of untreated mouse to subcutaneous tumour transplant (14 hrs)
Note oedema, hyperaemia and carbon labelling (→)

f Reaction to tumour transplant at 14 hours in mouse treated with zymosan
Note little oedema and little vessel labelling

g Reaction of untreated mouse to the subcuta neous injection of turpentine (lower part of picture) and tumour (upper part) at 14 hrs
Note inflammatory response at both injection sites

h Mouse treated with intravenous zymosan (14 hrs)
Note reduction in inflammatory response at both injection sites compared with untreated mouse (g)

i As g at 38 hours
Note inflammatory response at both injection sites

j Reaction to subcutaneous injection of turpen tine (lower part of picture) and tumour (upper part) at 38 hours in mouse treated with intra venous zymosan
Note reduced reaction around tumour trans plant compared with that in untreated mouse (i), but return of response at turpentine injec tion site compared to findings at 14 hrs (h)

8*

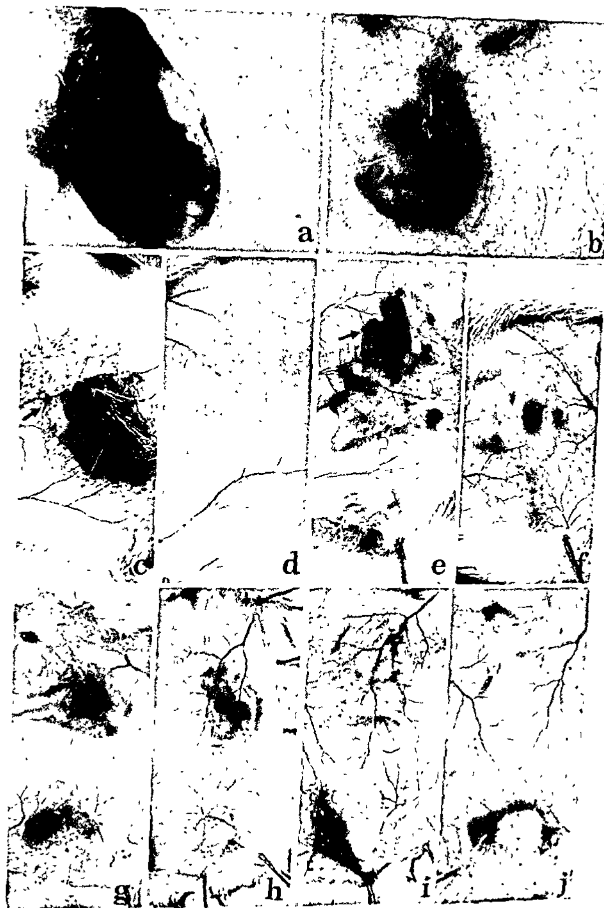

a
b
c
d
e
f
g
h
i
j

TABLE 2 *Size of the Subcutaneous Tumour Transplants (Mean ± S D) Related to Time after Transplantation and Treatment with Zymosan (see text to Exp 4)*

Treatment	Size of Transplant (mm) Time after transplantation (hrs)		
	14	14	38
Zymosan (i v)	20 ± 16	13 ± 8	18 ± 13*
Zymosan (i p)		18 ± 15	21 ± 22
Nil	33 ± 4	33 ± 6	30 ± 12

* 3 observations as one mouse died following treatment

decrease in the size of the lesion, significant in the second experiment at 14 hrs $0\,01 > P > 0\,001$) When zymosan was given intraperitoneally the scatter was greater

The lesions produced by turpentine injection did not differ statistically in size following treatment The difference was in the intensity rather than the extent of the reaction as described above

Attemps to 'recomplement' tumour bearing mice with fresh mouse serum met with little success Further experiments suggested that the intraperitoneal tumour may have taken up the new supply of complement

DISCUSSION

Mice with subcutaneous transplants of the Ehrlich carcinoma produce an acute inflammatory response to the tumour cells This is not characteristic of this tumour alone, but has been recorded with other mouse and rat tumours (*Sylven* 1967, *Spärck* & *Grost* 1969)

Coman & *Sheldon* (1946) believed that the hyperaemia they saw around transplants of Tumour 241 was due to cell proliferation, as it was also characteristic of embryonal tissue implants showing progressive growth and did not persist when these tissues started to regress Thus they link hyperaemia and tumour growth Recent work on the Ehrlich carcinoma has shown that the converse may also hold In the absence of an acute inflammatory response this tumour does not grow so well (*Hartveit* 1969) Histological studies have demonstrated that a factor other than cell proliferation is involved In the absence of hyperaemia and the acute inflammatory response that accompanies it, tumour cell proliferation (i e expansive growth) does occur, but infiltrative tumour growth is hampered (*Hartveit* & *Halleraker* 1970)

Plate II

a. Subcutaneous tumour transplant at 48 hours in mouse with a 12 day intraperitoneal transplant.
Note sharp edge of tumour H & E × 150

b As a
Note compact, morphologically intact tumour cells H & E × 600

c Subcutaneous tumour in mouse without other tumour at 48 hours
Note tumour cell necrosis compared with a and early infiltrative tumour growth in the *panniculus carnosus* H & E × 150

d As c
Note granulocyte infiltration between tumour cells H & E × 600

e Edge of tumour transplant 48 hrs after 0 5 ml 4 per cent aggregated gamma globulin (× 2)
Note small amount of cellular exudate between surface of transplant and *panniculus carnosus* H & E × 150

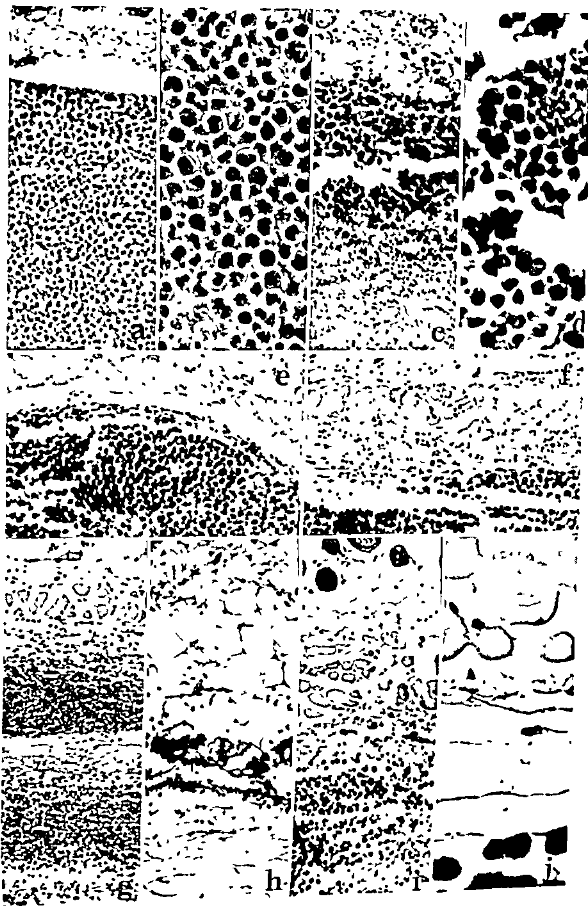
f As e, in mice given 0 25 ml 4 per cent aggregated gamma globulin (× 2)
Note oedema and host cell infiltration between edge of tumour and *panniculus carnosus* (compare e) H & E × 150

g Reaction of untreated mouse to subcutaneous turpentine (14 hrs)
Note inflammatory cell exudate H & E × 150
(N B The mice in these experiments also had 14 hour subcutaneous tumour transplants)

h Reaction to subcutaneous turpentine at 14 hours in mouse treated with intravenous zymosan
Note thin band of inflammatory cells H & E × 150

i Reaction of same untreated mouse as in g to subcutaneous tumour transplant
Note acute inflammatory response H & E × 150

j Reaction to subcutaneous tumour transplant at 14 hours, in mouse given intravenous zymosan
Note lack of inflammatory cells H & E × 600


a
b
c
d
e
f
g
h
i
k

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On this basis the postulate that the hyperaemia is due to cell proliferation alone cannot be accepted

Experiment 1 shows that the tumour cells fail to produce an acute inflammatory response in mice with late intraperitoneal transplants of the same tumour The subcutaneous tumours in these mice were clearly demarcated from the surrounding host tissue There was no tumour cell infiltration or inflammatory exudate formation The tumour cells were morphologically intact (The experiment does not show whether or not they were proliferating) In contrast the mice without intraperitoneal tumour showed a marked acute inflammatory response around the subcutaneous transplant and early infiltrative growth was present Many of the tumour cells in the centre of the transplants were necrotic and granulocyte infiltration was seen between them This tumour cell necrosis may be due to anoxia secondary to local circulatory disturbance This however seems unlikely as these tumour cells grow well under comparatively anaerobic conditions in the peritoneal cavity Further if failure of the acute inflammatory response around the subcutaneous tumour transplants in the mice with late intraperitoneal transplants were due to circulatory failure these transplants too should have necrotic centres

On the other hand the tumour cell necrosis seen in the untreated mice may have been the cause of the acute inflammatory response around the transplants due to the products of the dead cells and/or by products of the reaction that killed them e g complement mediated lysis see *Hartveit* 1965 a & b Its absence in mice with massive intraperitoneal tumour and in mice treated with other complement depleters supports this view

The finding that turpentine inflammation is also reduced by zymosan and in mice with late intraperitoneal transplants is in keeping with *Willoughby et al s* (1969) report that complement may also act as a mediator of inflammation of a non allergic type The evidence of a zymosan sensitive stage followed by a zymosan resistant stage in acute turpentine inflammation provided by the present experiments needs to be worked out in greater detail

If the availability of complement is a determining factor in the inflammatory response and subsequent infiltrative tumour growth the sex difference observed in our mice (*Hartveit* 1962) can probably be explained on this basis While there is no apparent difference in complement factor 1 in our mice (*Hartveit* 1964) or in mice of other strains investigated (see *Muller Eberhard* 1968) the complement factor 5 6 and 7 levels are lower in female mice than in males This could explain the sex differences seen in control mice in the present work and previously (*Hartveit* & *Halleraker* 1971) the slower tumour growth in female mice and their longer survival time (*Hartveit* 1962)

Zymosan is a polysaccharide a cell wall antigen that is widely distributed in nature first described by *Dungern* in 1900 Its inhibitory action at non toxic dosage on the growth of sarcoma 180 was reported in 1958 by *Bradner et al* The recent report by *Suzuki et al* (1969) that various polysaccharide components prepared from Bakers yeast (*S cerevisiae*) have tumour inhibitory properties supports this *Shear s* polysaccharide (1935) may have acted in a similar way and perhaps also Coley s toxins (see *Nauts et al* 1946) *Bradner et al* suggested that zymosan might stimulate some intermediate factor which in turn affects the tumor and/or the host in such a way that the tumor host relationship is changed in favor of the host and that the intermediate factor may be some function of the properdin system The present experiments suggest that this function may be the ability to inactivate complement factor 3 as the chief characteristic of the properdin system one of the so called non specific factors in immunity (*Nelson* 1960) is its ability to combine with zymosan and inactivate the third component of complement (see *Leon* 1960)

bedded in paraffin and stained Congo red (*Benn*

without any predisposing disease In this study, *the red green dichroism* of Congo Puchtler under the polarization microscopy was the criterion for the presence of amyloid

CASE REPORT

Case 1

Office cleaner died in 1969 at the age of 59 (see case 1, Meretoja 1969) The third of 4 siblings *The youngest brother* (aged 50), one of the 3 children (a 30 year-old son) of the patient and 7 other relatives were also affected by the syndrome concerned One affected cousin died of cancer of the stomach

Pruritus appeared at the age of 42 From the age of 43 she frequently had transient mild albuminuria Blood pressure was normal and intravenous urography revealed a moving right kidney At the age of 44 lattice dystrophy of the corneas was diagnosed

At the age of 43 polycythaemia vera was diagnosed She received 35 mCi ^{32}P during 11 years, *and busulphan for two years Bone marrow biopsy* at age 54 revealed the onset of myelofibrosis The diagnosis was confirmed later by repeated bone marrow punctures

At the age of 48 the right patellar reflex was absent for the first time. At the age of 59, bilateral upper facial paresis was observed, the reflexes of the lower limbs were absent, being markedly diminished in the upper limbs the vibration sensitivity was also diminished in the distal parts

At the age of 52, a large tumorous mass was observed in the bottom of the abdominal cavity At laparotomy the serosa and mesentery were found to be covered with white knobs The histopathological diagnosis was granulomatous inflammation Antituberculous treatment was instituted, and after 3 months the *patient was symptom free*

Four months before death her general condition deteriorated and two months later she became anaemic The blood count was Hb 5 7, erythrocytes 1 "9 thrombocytes 30 000 reticulocytes 1 1 per cent leucocytes 1 400, differential count myeloblasts 6 per cent, promyelocytes 6 per cent myelocytes 26 per cent metamyelocytes 10 per cent, lymphocytes 26 per cent, segmented 20 per cent, juvenile basophils and monocytes each 2 per cent. The blood count was suggestive of myelofibrosis, sternal puncture gave dry tap The patient was cachectic, the skin was yellowish and dry, and petechiae were observable The heart was enlarged, systolic murmur was heard The spleen and the liver were markedly enlarged One month before death, her temperature rose to 39° C, pain was felt in the thorax and shoulders, she suffered from diarrhoea and exhaustion Twenty days before death, supraventricular tachycardia developed Sixteen days before death, the leucocyte count was 7,600, and some days later 56 000 Before death, severe tachycardia was observed

Autopsy findings The heart weighed 470 g the left ventricle was hypertrophied Acute fibrinous pericarditis was observed The arterial system exhibited slight atherosclerotic changes. The right lung weighed 695 g, the left lung 480 g The weight of the liver was 1 980 g, chronic congestion was found The spleen weighed 595 g The general architecture was destroyed, the cut surfaces being homogenous and of reddish colour The marrow of the femur was red and hyperplastic. The kidneys weighed 120 g and 125 g, and did not exhibit *any macroscopical changes.*

Microscopical findings The bone marrow was typical of myeloid leukaemia no fat, only a few megakaryocytes, *and a diminished number of erythropoietic* cells, instead, the myelopoietic cells had replaced other marrow elements, and many imma*ture cell types were apparent The normal struc*ture of the spleen had entirely disappeared and had been replaced by leukaemic myeloid proliferation Leukaemic infiltrations were also *found in* the following organs liver, kidneys, lungs, heart muscle and pericardium Mild periarterial fibrosis was observed in the heart muscle Typical fibrinous inflammation was discernible in the pericardium. Small foci of neutrophils were found in the kidneys, and suggested acute pyelonephritis In Ziehl Neelsen stainings no acid fast bacilli were noted

Only changes typical of unspecific senile involution were noted in the brain Considerable accumulations of lipofuscin were evident in most cells The visual cortex appeared to be well preserved No obvious alterations were seen in the *Betz cells of* the precentral area, pyramidal tracts or spinal anterior horn neurones. In the cerebellum, the number of Purkinje cells appeared to be *slightly reduced In the spinal* cord, slight myelin degeneration was noted in the medial parts of the posterior columns, particularly at the cervical level. Numerous corpora amylacea *occurred in these* regions

A considerable loss of myelin sheaths of the facial nerves was apparent (*luxol fast blue*), many of these were very small in diameter A large amount of endoneural fibrosis existed, involving large numbers of cells with elongated *nuclei*. In the ischiadic, peroneal, sural and long thoracic nerves (Fig 1) and in the brachial plexus, similar

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HISTOPATHOLOGICAL FINDINGS OF FAMILIAL AMYLOIDOSIS WITH CRANIAL NEUROPATHY AS PRINCIPAL MANIFESTATION

Report on Three Cases

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A report is given of the histopathological findings in three cases of familial amyloidosis syndrome, presenting neurological and other symptoms, of which progressive facial nerve paralysis and lattice dystrophy of the cornea predominated The patients were two females 59 and 67 years of age, and one male of 79 years The first patient had polycythaemia vera and died of myeloid leukaemia, the second suffered from bulbar paralysis for several years and died of cerebral haemorrhage The third case died of pneumonia Amyloid was observed in nearly every organ studied It was localized mainly in the intima and the media of the arteries and capillaries, in most organs in the basement membranes as well Arteries totally obliterated by amyloid deposits were found in the parotic gland, the rectum and the spleen The glomeruli were markedly affected and the muscle tissue of the heart to a minor degree Moderate deposits of amyloid were also found in the perineurium and the endoneurium of most peripheral nerves studied However, clinically the cranial nerves were the most affected, and in one case, a branch of the facial nerve was totally replaced by amyloid Apart from the amyloid deposits in the meninges and arteries, only changes typical of unspecific senile involution were noted in the examination of the central nervous system The corneas and especially the anterior parts of the sclera were also affected

Several varieties of heredofamilial amyloidosis have been described Good reviews on the subject have been published by *Cohen* (1967) and *Schwartz* (1970) *Meretoja* (1969) reported 10 cases from 3 families with hereditary amyloidosis, commencing with lattice dystrophy of the cornea in the third decade Cranial nerve palsies mostly with upper facial paresis, began in about the fifth decade Other bulbar signs and minor peripheral neuropathy may appear later, even in the eighth decade Variable skin changes and internal symptoms were also observed Two of these 10 patients later died as also one from an earlier unpublished family The purpose of this paper is to report the autopsy findings and microscopical studies in respect of these three patients

MATERIAL AND METHODS

The autopsies were performed two days (case 1 and 2) and 8 days (case 3) after death The specimens were fixed in neutral formalin em-

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During the last two years, she received large quantities of medicines on account of the elevated blood pressure, urinary tract infection, cardiac insufficiency, obstipation and pains in the abdomen and thorax The haemoglobin values fell during the last year to 7 1, and the erythrocyte count to 2 4, the differential count was normal The urinary tract infection continued, and glucosuria was also noted The patient died at home after one day of unconsciousness and 4 days after she had left the local hospital

Autopsy findings The arms and legs were thin, attributable to muscular atrophy The meninges, the cerebellum and the hypophysis were normal The brain tissue adjacent to the right lateral ventricle was destroyed by a large haemorrhage, and the ventricular system of the brain was dilated and filled with clotted blood Cerebral haemorrhage was considered to be the cause of death. The heart weighed 265 g The coronary arteries revealed slight atheromatosis In the left pleural cavity were 200 ml of yellowish clear exudate The liver weighed 830 g, the cut surfaces were pale and yellowish no signs of congestion The kidneys were small, the surfaces were granular and the cortices thinned On the right side, a double pelvis and double ureter were observed, the ureters opened separately into the urinary bladder The spleen weighed 75 g

Microscopical findings In the brain, typical findings of recent haemorrhage were found In the heart muscle, periarterial fibrosis was the only pathological finding The kidneys were fibrotic due to chronic inflammation, and arteriosclerotic changes were observed as well The pancreas was somewhat fibrotic, and a medium sized artery inside the pancreas was totally occluded In the psoas major muscle considerable variation was apparent in the diameter of the muscle cells, and there were small collections of lymphocytes outside the vessels, no fat tissue or fibrosis was observed In the major branches of the facial nerve, the myelin sheaths exhibited moderate demyelination (luxol fast blue), and in many of the smallest branches, the loss of myelin sheaths was complete In the brachial, ischiadic and tibial nerves the changes were very similar to those in the facial nerves The perineurium was thickened Del Rio Hortega staining exhibited turgidity of the axon cylinders in some places

Stainings for amyloid The general pattern of amyloid deposits corresponded to the findings in the case 1 (Table 1) The intimal and medial parts of both small and large arteries were the locations most obviously affected (Fig 3) The aorta and common iliac artery contained amyloid in varying intensity in the intima and media and sometimes also in the adventitia near the media The fibrous tissue around the arteries of the myocardium contained amyloid, and in some areas, small amyloid deposits were observed in the muscle tissue independent of the arteries Very minute amyloid deposits were observable in the walls of some alveoli of the lungs, and the visceral pleura contained small fibrillar deposits of amyloid beneath the mesothelial layer Deposits of amyloid in the glomeruli of the kidneys were clearly visible (Fig 4), and also around some of the tubules in the cortex The basal membranes of both the acini and the ducts of the parotic gland were affected The amyloid had replaced the acinar tissue in some areas, and amyloid was also observable outside the ducts One artery was almost totally obliterated by amyloid masses The basal membranes of the follicles in the thyroid gland contained little amyloid In the muscular layer of the gallbladder (operation specimen from 1963), remarkable deposits of amyloid were found The basal membranes around the eccrine sweat glands of the skin were affected, and also to a minor extent around the sebaceous glands

Diffuse amyloid deposits were observed in the leptomeninges and dura of the brain and spinal cord, as in case 1 The arteries and the capillaries were moderately affected, but the nervous system itself was free from amyloid Some of the branches of the facial nerves were rather well preserved, with only minimal perineural amyloid deposits, whereas others had an abundance of amyloid also in the endoneurium The endoneural deposits were often bound to the perineurium In other nerves studied (Fig 5), the thickened perineurium contained amyloid, in the endoneurium, the amyloid existed as thin fibrillary deposits of a curled appearance, and only in few places did the deposits form major accumulations

The corneas and other parts of the bulbus showed changes similar to those in case 1 (Fig 6)

Case 3

Farmer, died in 1970 at the age of 79 The family lived in the same rural area as the families of the two cases above The pedigrees were traced to the eighteenth century and no connection was found The sister and 3 other relatives of the patient were affected by the syndrome concerned here

Glaucoma simplex was noted when the patient was 60 years old, and a bilateral operation was made The left eye became blind when he was 64, and the right eye 14 years later Lower facial paresis was diagnosed at the age of 70 Between the age of 72 and 75 four operations were made for palpebral ectropium

The patient who suffered from vertigo was admitted, at the age of 74, to the Poor Law Institution, the blood pressure was normal Transient albuminuria was noted once

Five months before his death, the patient was

changes were observed The perineurium often appeared to be thickened

Stainings for amyloid The amyloid was pink with the *haematoxylin and eosin stain*, khaki with the van Gieson technique, PAS positive, and showed methyl violet and toluidine blue metachromasia, and thioflavine T fluorescence The results of these methods were, however, somewhat uncertain and difficult to interpret The results of the stainings for amyloid with Congo red by *Puchtler et al* are presented in Table 1

The general pattern of amyloid distribution in the various organs was as follows small and medium sized arteries were nearly always affected, as were also arterioles, and in many instances the capillaries as well The location of the amyloid in the arterial wall was predominantly in the intima and the media. The adventitia was mostly negative, and *only the larger arteries (e g the aorta and the* common iliac artery) had small amyloid deposits in their adventitial parts The veins were often negative Some larger veins (e g femoral vein and the veins in the capsule of the thyroid) showed minimal deposits along the lamina elastica interna and in the media and the adventitia.

In the heart, the fibrotic tissue around some arteries contained fibrillar or clumpy deposits of amyloid In some places the muscle cells had been replaced by amyloid containing amorphous material, however, the major part of the myocardium *was free of amyloid The mesothelium of the peri*cardium was accompanied by a thin layer of amyloid Lung parenchyma was negative but the visceral pleura exhibited small streaks of amyloid beneath the mesothelial layer The glomeruli of the kidneys were markedly affected granular or more diffuse deposits of amyloid were apparent in every glomerulus The basement membranes of the tubuli were also affected, but much less than the glomeruli The basal membrane of some excretory ducts of the parotic gland had been thickened by *amyloid containing material, and in some places* the parenchyma had also been replaced by amyloid A large artery inside the parotic gland and near the facial nerve was completely occluded by amyloid masses In the thyroid gland the connective tissue around the vessels and follicles contained some amyloid

Diffuse fibrillary amyloid deposits were observed in the leptomeninges and dura of the brain and spinal cord The arteries and the capillaries of the central nervous system were affected in a way *similar to* that *in* other parts of the body The nervous tissue itself was free from amyloid

The perineurium and the endoneurium of the peripheral nerves provided another common site of amyloid deposits The changes were most remarkable in the facial nerves in places, the major part of the transverse sections was *filled by diffuse* fibrillary amyloid deposits, infiltrating the endoneurium One branch of the upper facial nerve was almost entirely destroyed by amyloid (Fig 2) In the other peripheral nerves studied, the thickened perineurium was infiltrated by *amyloid deposits* Changes in the endoneurium were most pronounced in the immediate neighbourhood of the perineurium Small nerve branches seemed more affected than large ones, but total destruction by amyloid was not noted In the mid thoracic portion of the truncus sympaticus, amyloid deposits were seen in the perineurium and the endoneurium the changes were less marked in the superior cervical ganglion

In the cornea, a thin layer of amyloid deposits were situated under the Bowman s membrane and larger circumscribed masses deeper in the stroma The arteries of the eye were moderately affected *In the anterior part of the sclera, in the area of* the anterior ciliary arteries the deposits were more pronounced and formed great accumulations Amyloid deposits were also found under the epithelium of the conjunctiva

Case 2

Housewife, died in 1969 at the age of 67 (see case 10 Meretoja 1969) The tenth of 11 siblings 4 of whom were affected by the syndrome concerned One of her 4 children (a 42 year old son) and 6 other relatives were also affected One of these had cancer of the tongue which was cured with x ray therapy

At the age of 51 she had left sided hemiplegia from which she recovered sufficiently to enable her to walk but later, from the age of 53 she suffered from bulbar paralysis with progressive tiredness At the age of 58 neurological examination revealed affection of the cranial nerves V and VII–XII Neurological examination 8 years later showed only little progression The patient was still cachectic and the small muscles of the hands were *atrophic The muscles of the face* were also atrophic There were fibrillations in the tongue and the lower muscles of the face The speach was aphonic, the swallowing was difficult and the hearing was impaired as well as the visual acuity because of corneal dystrophy The left Babinski reflex was positive no spasticity *existed The ex*tremities of the left side were ataxic and the left upper limb made athetotic movements The patient could walk with difficulty when lead by hand

From the age of 42 she had a high blood pressure Transient albuminuria was noticed for the first time when she was 57 years *old Cholecystec*tomy because of gall stones was performed when she was 61 Two years later, biopsy of the enlarged cervical lymph nodes revealed exudative tuberculosis with caseation and tubercles and tuberculostatic therapy was instituted

TABLE 1 *(Cont)*

Organ or tissue	Case 1	Case 2	Case 3
Skin		BM of sweat and sebaceous glands +	BM of epidermis sweat and sebaceous glands +, arrector pili muscle +
M psoas major		—	—
Tendon			peritendinous tissue +
Tongue			BM of salivary glands and ducts +, connective tissue +, muscle cells —
Tonsils		subepithelial part ±	
Esophagus			—
Stomach	muscular layer ±, mucosa —	as in case 1	as in case 1
Intestine	—	—	—
Rectum	muscular layer ±, mucosa —		as in case 1
Gall bladder		*muscular layer + +* *	—
Meninges	dura + leptomeninges +	as in case 1	as in case 1
Brain tissue	—	—	—
Facial nerve	perineurium + +, endoneurium + + (Fig 2)	perineurium +, endoneurium +	as in case 2
Other nerves	perineurium +, endoneurium + (Fig 1)	as in case 1 (Fig 5)	as in case 1
Cornea	stroma + under Bowman s membrane +	as in case 1 (Fig 6)	as in case 1
Medulla of the femur		—	—

+ + heavily or moderately affected + – slightly affected, ± only minute deposits — = negative
BM basement membrane The arteries and arterioles in every organ are positive and have not been mentioned separately
* Operation specimen from 1963

examined with relation to the syndrome He was cachectic There were cigarette paper scars on the knees and the skin of the legs was scratched because of irritation The linings of the abdominal wall were thin and the abdomen hung balloon like The squeezing of the hand was normal The deep sensations of the upper extremities were diminished The muscles of the face were extremely atrophic bilateral upper and lower facial paresis were observed and he was unable to close his mouth because of the hanging lower lip The conjunctivae were injected because of bilateral lagophthalmus The corneae were covered by central leucomas and lattice lines were visible only at the periphery of the corneas The hearing [illegible]

[illegible] therapy proved to be without effect

Autopsy findings The skin on the scalp was atrophic. Arteriosclerosis was moderate except in the coronaries where many severely stenotic segments were noticed An old infarction was seen in the left ventricle of the heart and the mitral valve was calcified at its base Both pleural cavities revealed 300 ml of clear exudate Bilateral, far ad

TABLE I *Results of the Staining of Amyloid with Congo Red by Puchtler et al*

Organ or tissue	Case 1	Case 2	Case 3
Aorta and large arteries	intima +, media + +, adventitia ±	as in case 1	as in case 1
Small arteries	intima +, media + +, adventitia —	as in case 1 (Fig 3)	as in case 1
Capillaries	+ +	+ +	+ +
Large veins	lamina elastica interna +, media and adventitia ±	as in case 1	as in case 1
Small veins	—	—	—
Heart	muscle cells ±, pericardium +, periarterial fibrous tissue + +	as in case 1	as in case 1
Epiglottis, larynx and trachea			BM of the glands +
Lungs	alveoli —, bronchi —, pleura ±	alveolar BM +, bronchi —, pleura ±	BM of alveoli, bronchi and mucosal glands +, pleura ±
Liver	—	—	—
Spleen	—	—	parenchyma —, capsule ±
Kidneys	glomeruli + +, BM of tubules +	as in case 1 (Fig 4)	as in case 1
Hypophysis		adeno- and neuro hypophysis +	as in case 2
Thyroid	BM of follicles ±, connective tissue —	as in case 1	BM of follicles + connective tissue +
Adrenals	—	—	medulla —, cortex +, capsule +
Pancreas	—	—	—
Parotic gland	BM of acini and ducts +, parenchyma +	as in case 1	as in case 1
Uterus and ovary		—	
Testis			BM of ducts +, tunica albuginea ±
Epididymis			BM of ducts + +
Seminal vesicle			BM +
Prostate			BM ±, corpora amylacea +
Corpus cavernosum			±
Lymph nodes		—	capsule +

vanced *pneumonia* and purulent bronchitis were the main findings and they were considered to be the cause of death Haemorrhagic infarction caused by a small embolus was observed in the lingula of the lower lobe of the left lung Other findings worth mentioning were *the enlarged* lymph nodes laterally on the left side of the neck calculi in the *gall bladder* and the common bile duct (no signs of biliary obstruction), and a grey homogenous area (Ø 0 5 cm) in the medulla of the right adrenal gland The heart weighed 420 g the liver 1 160 g the kidneys 120 and 125 g

Microscopical findings The specimens from the lungs revealed extensive purulent pneumonia with [illegible]

found The Ziehl Neelsen stains were negative The arteries revealed sclerotic changes and in the arteries of the tongue the quadriceps femoris muscle and the thyroid gland mediasclerosis of Mönckeberg type was observed The changes in the peripheral nerves were similar to those in cases 1 and 2 although total destruction of nerve branches *could not be demonstrated*

Stainings for amyloid The results of the staining for amyloid resembled largely the findings in cases 1 and 2 (see Table I) The amyloid deposits in the arteries and the arterioles were more pro*minent than in cases 1 and 2* The amyloid in the large arteries (e g the aorta the carotid and the femoral arteries) were localized especially in the outer part of the media where the deposits formed a clear-cut zone Also the veins were positive in many instances The nerves were affected to a minor extent and amyloid was shown to be present *mainly in the perineurium of the peripheral nerves* The brain tissue and the medulla were negative There was amorphous material around the sweat glands epididymal ducts glands of the mucosa of the respiratory tract and the acini of the salivary *glands which* proved to be amyloid The corpora amylacea in the prostatic acini were positive

DISCUSSION

The type of amyloidosis beginning with cranial nerve *symptoms* predominantly facial paresis and corneal dystrophy, is a third clearcut type of *familial* amyloidosis with polyneuropathy The Portuguese type described by *Andrade* (1952) *affects* primarily the lower limbs and also causes digestive and sexual disturbances, and death The other type reported by *Rukavina et al* (1956), comprises peripheral neuropathy chiefly affecting the arms, carpal tunnel syndrome, cardial insufficiency, vitreous opacities and [illegible] hepatome- [illegible]

logical types of amyloidosis, based upon the initial deposition of amyloid in relation to *reticular or to collagen fibres.* In perireticular amyloidosis the depositions start in the intima *and* spread to the tunica media Amyloid involves also the reticulin fibres which support *the parenchymal cells, renal glomeruli,* liver and adrenals The pericollagen type involves connective tissue *and starts from the* tunica adventitia of blood vessels Parenchymal replacement is also frequent (*Heller et al* 1964) The amyloidosis syndromes described by Andrade and Rukavina et al belong to the pericollagen type

In our cases, the amyloidosis was apparently of the perireticulin type Large, isolated amyloid deposits were only occasionally observed and the amyloid was most frequently located as thin fibrillar streaks The localization of amyloid in the intima and the media of the arteries, as well as along the basement membranes is typical for the perireticulin type

The histopathological *findings* in the peripheral nerves corresponded well with the *clinical findings* the amyloid deposits were most extensive in the facial nerves Facial paresis was *observed in all cases The other* neurological symtoms suggesting peripheral *neuropathy may, at least partly,* be caused by endoneural amyloidosis

The arteries were affected in nearly every organ, but symptoms which could be explained by amyloid deposits in the walls of the vessels cannot be differentiated with certainty from arteriosclerotic symptoms

Both of our female cases had proteinuria for many years In case 2, this may partly be attributable to the urinary tract infection, but at the time of appearance of the symptom, no infection was observed The amyloid deposits in the glomeruli in both cases may well be the cause of the proteinuria



Fig 1 Long thoracic nerve, case 1 Marked demyelination Luxol fast blue, × 250 All photos Agfacolor 20 DIN

Fig 2 Facial nerve, case 1 The smaller of two branches is almost totally replaced by amyloid deposits which destroy the inner structure of the nerve Congo red by Bennhold, × 40

Fig 3 Arteries of the thyroid gland, case 2 The medial and the intimal layers have been replaced by amyloid masses, which is shown by green dichroism Congo red by Puchtler et al, crossed polars, × 16

Fig 4 Kidney case 2 The glomeruli have been replaced by amyloid tissue, some minor deposits are also scattered along the arterioles Congo red by Puchtler et al, × 20

Fig 5 Tibial nerve case 2 Moderate amyloid deposits in the perineurium and the endoneurium are shown by green dichroism Congo red by Puchtler et al, crossed polars, × 80

Fig 6 Cornea, case 2 Amyloid deposits are situated as a thin layer under the Bowman's membrane and as circumscribed masses deeper in the stroma representing transverse sections of the clinically visible lattice lines Congo red by Puchtler et al, × 40

vanced pneumonia and purulent bronchitis were the main findings, and they were considered to be the cause of death. Haemorrhagic infarction caused by a small embolus was observed in the lingula of the lower lobe of the left lung. Other findings worth mentioning were the enlarged lymph nodes laterally on the left side of the neck, calculi in the gall bladder and the common bile duct (no signs of biliary obstruction), and a grey homogenous area (Ø 0.5 cm) in the medulla of the right ... The heart weighed 420 g, the liver ...

... abscesses and aspirated material in some alveoli.

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Stainings for amyloid. The results of the stainings for amyloid resembled largely the findings in cases 1 and 2 (see Table 1). The amyloid deposits in the arteries and the arterioles were more prominent than in cases 1 and 2. The amyloid in the large arteries (e.g. the aorta, the carotid and the femoral arteries) were localized especially in the outer part of the media where the deposits formed a clear-cut zone. Also the veins were positive in many instances. The nerves were affected to a minor extent and amyloid was shown to be present mainly in the perineurium of the peripheral nerves. The brain tissue and the medulla were negative. There was amorphous material around the sweat glands, epididymal ducts, glands of the mucosa of the respiratory tract and the acini of the salivary glands which proved to be amyloid. The corpora amylacea in the prostatic acini were positive.

DISCUSSION

The type of amyloidosis beginning with cranial nerve symptoms, predominantly facial paresis and corneal dystrophy, is a third clearcut type of familial amyloidosis with polyneuropathy. The Portuguese type described by *Andrade* (1952) affects primarily the lower limbs and also causes digestive and sexual disturbances and death. The other type reported by *Rukavina et al.* (1956) comprises peripheral neuropathy chiefly affecting the arms, carpal tunnel syndrome, cardial insufficiency, vitreous opacities and gastro-intestinal symptoms with hepatomegaly and splenomegaly.

Missmahl (1959) has recognized two histological types of amyloidosis, based upon the initial deposition of amyloid in relation to reticular or to collagen fibres. In perireticular amyloidosis the depositions start in the intima and spread to the tunica media. Amyloid involves also the reticulin fibres which support the parenchymal cells, renal glomeruli, liver and adrenals. The pericollagen type involves connective tissue and starts from the tunica adventitia of blood vessels. Parenchymal replacement is also frequent (*Heller et al.* 1964). The amyloidosis syndromes described by Andrade and Rukavina et al. belong to the pericollagen type.

In our cases, the amyloidosis was apparently of the perireticulin type. Large, isolated amyloid deposits were only occasionally observed and the amyloid was most frequently located as thin fibrillar streaks. The localization of amyloid in the intima and the media of the arteries, as well as along the basement membranes, is typical for the perireticulin type.

The histopathological findings in the peripheral nerves corresponded well with the clinical findings: the amyloid deposits were most extensive in the facial nerves. Facial paresis was observed in all cases. The other neurological symtoms suggesting peripheral neuropathy may, at least partly, be caused by endoneural amyloidosis.

The arteries were affected in nearly every organ, but symptoms which could be explained by amyloid deposits in the walls of the vessels cannot be differentiated with certainty from arteriosclerotic symptoms.

Both of our female cases had proteinuria for many years. In case 2, this may partly be attributable to the urinary tract infection, but at the time of appearance of the symptom, no infection was observed. The amyloid deposits in the glomeruli in both cases may well be the cause of the proteinuria.



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DNA-SYNTHESIS OF LYMPHOCYTES IN REGENERATING LIVER

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There are numerous lymphocytes within the liver It seems as if part of the recirculation of lymphocytes takes place via the liver itself and its lymph The functional significance of the lymphocytes of the liver is unknown The general aim of the present investigations was to gain some more information about the liver lymphocytes The particular question asked was Are lymphocytes within the liver stimulated to proliferate simultaneously with the liver cells during liver regeneration? Liver regeneration was *induced with* CCl_4 *in mice and* DNA synthesis was studied with the aid of H_3 thymidine labelling and autoradiography The question could be answered affirmatively Yes, the lymphocytes were stimulated to DNA synthesis either by a humoral factor at a distance *or in situ The functional significance of the findings are discussed*

There are numerous lymphocytes in the hepatic interstitium, mainly about the biliary ducts and the branches of the portal vein but also diffusely spread in the parenchyme (13 20, 22) This lymphocyte infiltration is not constant but changes under different conditions (11 12 19)

There is to day a general agreement about a large scale recirculation of lymphocytes from blood to lymphoid tissues and back to blood It has been argued that part of this recirculation of lymphocytes takes place via the hepatic lymph nodes in rabbits (6), and in guinea pigs (7 8), and via the hepatic lymph in dogs (2) It has recently been demonstrated that most of the diffusely spread lymphocytes of the mouse liver are long living and thus seem to have the same age distribution as have blood lymphocytes (14) This finding indicates that at least part of the recirculation of lymphocytes via the hepatic lymph and the hepatic lymph nodes also takes place via the liver itself

The functional significance of a lymphocyte recirculation via the liver has been discussed (5, 8) It is tempting to postulate that the large number of lymphocytes in the liver, more than 10 per cent of the cells of this large organ represents a readily mobilizable reserve of immunocompetent cells.

The general aim of the present investigation was to gain some more information about the lymphocytes within the liver

The proliferation of lymphocytes within the thymus is larger than in the other lymphatic organs (1, 16) It is assumed that the micro-environment of this lympho epithelial organ has this stimulating effect on the lymphocytes reaching there The bursa Fabricii, another lympho epithelial organ, is also characterized by a high rate of proliferation of its lymphocytes (10, 21) Again the epithelial component of this organ has been made responsible for this

Received 16 1 71

The stromal circumscribed masses of amyloid in the cornea corresponded with the clinically visible lattice lines These have been earlier considered to be stromal nerves (*Vrabec* 1957), and proved to be of amyloid origin (*Seitelberger* & *Nemetz* 1961)

Two out of eight patients over 40 years in the earlier series of Meretoja and case 3 of this report had glaucoma simplex The 3 cases described now had considerable accumulations of amyloid in the sclera in the area of the anterior ciliary arteries This was possibly the aetiologic factor for glaucoma in the oldest patients since it prevented the normal outflow

In case 1, amyloid was observed at re-examination of the biopsy specimens (peritoneum and omentum) on the basis of which the initial diagnosis of tuberculosis had been established On re examination of case 2, the gall-bladder, which was removed two years before tuberculous lymphadenitis was diagnosed, was also found to be clearly positive for amyloid These facts exclude the possibility that the amyloidosis in these cases was secondary to tuberculosis The familial occurrence of the disease and the ubiquitous histological distribution of the amyloid also favour the opinion that the amyloidosis was primary In case 3 histologically active tuberculosis was observed at autopsy, but the localization of the amyloid was not that of secondary amyloidosis

The three cases reported by *Klaus et al* (*1959*) possibly represent the same syndrome as that described here They reported 3 sisters in Czechoslovakia who presented with a bulbar paralytic form of amyotrophic lateral sclerosis, corneal dystrophy of lattice type, and cutis hyperelastica One of these patients died of adenocarcinoma of the uterus They did not describe the peripheral nerves and no stainings for amyloid were performed The changes in these patients were so advanced that a possible initial facial nerve paresis was hidden No neuropathological alterations characteristic of amyotrophic lateral sclerosis were noted in the cases now presented

The disease described here seems to be rather common in Finland So far, one of the authors (J M) has encountered 90 cases in 21 families

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We wish to thank Dr *Matti Haltia* M D for helping us in the interpretation of the neuropathological findings, and Dr *Veikko Mak a*, M D for performing the autopsy in the ca e 1

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second possibility is that lymphoid cells become both stimulated and *synthesize* DNA while in the liver Further experiments are needed to judge in this question

It has just been reported *that the number* of antibody forming cells detected as hae-

is significantly increased after partial hepatectomy (18) The mechanism behind this *could be a humoral* factor acting upon the virgin lymphocytes or primed lymphocytes in the spleen and lymph nodes (17) It could also be that lymphocytes stimulated within the liver itself are involved

The high mitotic activity of the lymphocytes of the two central lympho-epithelial organs, *thymus and bursa Fabricii, was men*tioned in the introduction The question asked there *Are the lymphocytes within the* liver stimulated to proliferate simultaneously with the liver cells during liver regeneration? can now be answered affirmatively Yes, either by a humoral factor at a distance or *in situ This does not mean that the liver* shall be looked upon as a potential central lympho-epithelial organ The age distribution amongst the liver lymphocytes (14) similar to that of blood lymphocytes, points in quite other *directions*

The findings are of interest from another point of view namely for the interpretation of an experiment by *Hard & Kullgren* (9) C_3H mice made chimeric by lethal × irradiation followed by injection of $(C_3H \times T_6)F_1$ spleen cells, were later stimulated by CCl_4 to produce a burst of liver cell mitosis Cytogenetic studies of the regenerating livers of 11 chimeras identified 89 per cent of the cells as donor type by presence of the distinctive T_6 marker As is pointed out by the authors themselves there is a gap between the histological and cytological studies It is impossible to identify the T_6 marker chromosome in tissue sections of the liver, nor can one determine the type of cell in mitosis in the cytogenetic studies *Hard & Kullgren* writes "Although there is a minimal degree of inflammatory *-cell infiltration* and littoral-cell proliferation, it is possible that these were the only cells to have survived the preparative procedures" They mention as a possibility the interpretation that the hepatic parenchymal cells of the host have taken up genetic material from the donor spleen cells, one whole chromosome at least The present finding of increased proliferation of lymphoid cells during liver regeneration weakens the support *for this iconoclastic interpretation*

The work was supported by the Swedish Medical Research Council

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The liver is certainly an epithelial organ in which lymphocytes come into close contact with epithelial cells The question was then asked Are lymphocytes within the liver stimulated to proliferative simultaneously with the liver cells during liver regeneration? In order to answer that question we have studied the DNA synthesis of the lymphocytes diffusely spread in the liver during regeneration following the administration of carbon tetrachloride ($C Cl_4$) The DNA synthesis was studied with the aid of H_3 thymidine and found to be increased

MATERIAL AND METHOD

40 mice, obese hyperglycaemic strain originating from the R B Jackson lab, Bar Harbour, Main, USA, genotype OB, weighing between 20 and 29 g were used They were all labelled by i v injections of 0 5 uC H_3 thymidine/g bodyweight (spec act 6 7 C/mM, Schwarz Bioresearch, diluted with 0 9 per cent saline to a concentration of 500 uC/cc before injection) All injections were made between 9 and 10 a m and all animals were killed 30 minutes later

The animals were divided into 5 groups with 8 animals in each Group I was treated with $C Cl_4$ 24 hours before labelling Groups II–IV were treated the same way 48, 72 and 96 hours, respectively, before labelling Group V was the untreated control group

The $C Cl_4$ used was a 40 per cent solution in sesam oil It was injected subcutaneously and each animal received 0 1 cc per 20 g bodyweight A piece of the liver of the animals was fixed in 4 per cent carbonate buffered formalin After dehydration the pieces were cleared in xylene After embedding in paraffin sections were cut at 5 u Autoradiographs were prepared with liquid emulsion technique described by *Back* (3) The slides were exposed for 14 days and developed with Kodak D 19B for 2 minutes shortly dipped in water and fixed with Kodak acid fixer for 10 minutes After drying in front of a fan the slides were stored until stained with haematoxylin according to *Mayer* (15)

Only slides with reasonably low background were used for counting the number of labelled lymphocytes or lymphoid cells Every such cell (with a round, chromatin dense nucleus) with four or more than four grains was regarded as labelled One hundred of the diffusely spread lymphoid cells from each animal were counted Collections of lymphocytes around the vessels were avoided All counting work was done by a specially trained technician who was unaware of the design of the experiment

RESULTS

The mean percentage of labelled lymphoid cells in the liver was about 0 5 in the control group and in the animals killed 24 hours after the administration of $C Cl_4$

48, 72 and 96 hours after the administration there was a manyfold increase of labelled lymphoid cells in the liver See Table 1

TABLE 1 *Per Cent Labelled Lymphoid Cells in the Liver at Different Times after the Administration of $C Cl_4$ H_3 thymidine Injected 30 Minutes before Killing the Mice*

	Control	24 hours	48 hours	72 hours	96 hours
	0	0	2	4	2
	0	1	3	3	3
	1	0	1	3	4
	0	0	2	3	5
	1	1	3	5	3
	0	0	3	2	1
	1	0	2	3	2
	2	1	2	2	3
Mean	0 6	0 4	2 3	3 1	2 9

DISCUSSION

During the course of this investigation we came to know about the work of *Craddock* (4) in which he demonstrated that 50 per cent resection of the liver triggers DNA synthesis not only in the regenerating liver cells but also in lymphatic organs Such a stimulation of DNA synthesis in lymphatic organs could of course also be a parallel phenomenon to the liver regeneration following $C Cl_4$ administration It is less probable that lymphoid cells can take up the label and migrate into the liver during the short labelling time 30 minutes It is, however, quite possible that lymphoid cells outside the liver become stimulated to DNA synthesis migrate into the liver, and synthesize DNA while there A

second possibility is that lymphoid cells become both stimulated and synthesize DNA while in the liver Further experiments are needed to judge in this question

It has just been reported that the number of antibody forming cells detected as haemolytic plaque forming cells, or immune rosette forming cells in the spleen and lymph nodes of rats immunized with sheep red cells is significantly increased after partial hepatectomy (18) The mechanism behind this could be a humoral factor acting upon the virgin lymphocytes or primed lymphocytes in the spleen and lymph nodes (17) It could also be that lymphocytes stimulated within the liver itself are involved

The high mitotic activity of the lymphocytes of the two central lympho-epithelial organs, thymus and bursa Fabricii, was mentioned in the introduction The question asked there Are the lymphocytes within the liver stimulated to proliferate simultaneously with the liver cells during liver regeneration? can now be answered affirmatively Yes, either by a humoral factor at a distance or in situ This does not mean that the liver shall be looked upon as a potential central lympho epithelial organ The age distribution amongst the liver lymphocytes (14) similar to that of blood lymphocytes, points in quite other directions

The findings are of interest from another point of view namely for the interpretation of an experiment by *Hard & Kullgren* (9) C H mice made chimeric by lethal X-irradiation followed by injection of $(C_3H \times T_6)F_1$ spleen cells were later stimulated by CCl_4 to produce a burst of liver cell mitosis Cytogenetic studies of the regenerating livers of H chimeras identified 89 per cent of the cells as donor type by presence of the distinctive T_6 marker As is pointed out by the authors themselves there is a gap between the histological and cytological studies It is impossible to identify the T_6 marker chromosome in tissue sections of the liver nor can one determine the type of cell in mitosis in the cytogeneic studies *Hard & Kullgren* writes "Although there is a minimal degree of inflammatory-cell infiltration and littoral-cell proliferation, it is possible that these were the only cells to have survived the preparative procedures" They mention as a possibility the interpretation that the hepatic parenchymal cells of the host have taken up genetic material from the donor spleen cells, one whole chromosome at least The present finding of increased proliferation of lymphoid cells during liver regeneration weakens the support for this iconoclastic interpretation

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A CYTOCHEMICAL STUDY OF LIVER PROLIFERATION AND EXPERIMENTAL LIVER DISORDERS IN THE RAT

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The cytochemical findings in cytological preparations of rat liver following partial hepatectomy were compared with those observed in connexion with α naphthyl isothiocyanate (ANIT) and thioacetamide (TAA) intoxication In conjunction with liver regeneration after partial hepatectomy, the main findings were extensive bile canalicular branching, as revealed by staining for naphthylamidase, cytoplasmic fatty vacuolization, enlargement of nucleoli, and an increase in succinic dehydrogenase and β glucuronidase activities After a single injection of TAA, a biphasic proliferation pattern seemed to be demonstrable, after a primary stage of bile canalicular branching, hepatocellular damage was noted, whereafter a second stage of canalicular branching followed During ANIT treatment bile canalicular dilatation was the main feature The results of the study seem to offer useful cytochemical criteria of hepatocellular proliferation

It has recently been shown (*Wasastjerna et al* 1970 a, b), that both human and experimental liver diseases are reflected as bile canalicular changes, which can be visualized in cytological preparations by the naphthylamidase staining method of *Wasastjerna* (1969) Difficulty in interpretation of the findings arises in questions of complex liver affections such as liver cirrhosis, in which a proliferative component needs to be considered in addition to hepatic damage It thus seemed important to establish the cyto chemically demonstrable characteristics of the regenerating liver

In the present study, liver regeneration after partial hepatectomy was taken as a model for hepatic proliferation In addition to sham-operated and untreated controls, animals treated with thioacetamide (TAA), and α naphthyl isothiocyanate (ANIT) were included in the series as reference material, TAA brings about a cytological picture of hepatitis when administered to guinea pigs (*Wasastjerna et al* 1970 a), whereas ANIT has been applied to induce intrahepatic cholestasis in rats (*Desmet et al* 1968)

Received 13 i 71

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MATERIAL AND METHODS

The animals used were young female albino rats, weighing from 130-180 g

Ten animals were subjected to partial hepatectomy in ether anaesthesia, by application of the method of *Higgins & Anderson* (1931) Through a midline incision reaching approximately 3 cm distally from the xiphoid process of the sternum

the median liver lobe and the left lateral liver lobe were delivered, ligated with coarse silk and excised thus leaving within the peritoneal cavity the right lateral lobe and the caudate lobe The incision was closed with two layers of silk sutures the muscles with the peritoneum and the integument being closed separately Asepsis was maintained throughout the operation Two animals were killed after 20 24 36 48 and 72 hours respectively Two control animals were subjected to the same procedures as the hepatectomized animals except that ligation and the removal of liver tissue were omitted The control animals were killed 2 days after the operation

TAA intoxication was induced by a single intraperitoneal injection of 200 mg TAA/kg body weight (b w) as a 2 per cent saline solution The animals were killed after 1, 2 4 6 and 9 days respectively, two on each occasion Two control animals were given a single intraperitoneal injection of 1 ml saline and were killed 4 days later

ANIT intoxication was induced by oral administration of the drug in daily doses of 45 mg/kg b w The drug was suspended in olive oil to yield the daily dose in 0 1 ml and was administered by gastric catheterization in light ether anaesthesia Two control animals were given daily doses of 0 1 ml olive oil by the same method The ANIT treated animals were killed in pairs after 1, 2 4, 6 8 10 and 15 days of treatment The control animals were killed after 15 days

Five untreated animals served as normal controls Throughout the experiment the animals were killed by decapitation

Thin slices were cut from the liver and imprints were prepared on glass slides by gently bringing them into contact with the cut liver surface In addition to staining according to the May Grunwald Giemsa (MGG) method preparations were stained for the demonstration of naphthylamidase (N ase) by a modification of the method of *Rutenburg* & *Rosales* (1966) of β glucuronidase (β gluc) according to *Lorbacher Yam* & *Ouamnor* (1968) and of succinic dehydrogenase (SDH) by the method of *Goebel* & *Puchtler* (1955) Staining for the demonstration of fat was occasionally effected with Oil red O as the staining agent (*Lillie* 1965)

Before fixation and staining the preparations were stored for 24 hours at +4° C Staining for N ase was preceded by fixation for 3 minutes in a cold solution of equal amounts of acetone and chloroform The preparations to be stained for the demonstration of β gluc were fixed in cold 20 per cent acetone formalin for 1 minute for SDH the preparations were fixed for 15 seconds in room tempered, 60 per cent acetone (*Quagliano* & *Hayhoe* 1960)

The stained slides were coated with Apathy s gum syrup and a covering slip and were examined within 2 days

RESULTS

MGG The cytoplasm of normal hepatocytes was unevenly basophilic, and appeared cloudy (Fig 1) The nuclei were round to oval, and contained 1–3 small distinct nucleoli Pigment granules were not discernible in the cells Mitoses were not observable

After hepatectomy the hepatocyte cytoplasm exhibited marked vacuolization (Fig 2), the contents of the vacuoles were fat as revealed by fat staining In addition, after 2 and 3 days the cytoplasm presented a more organized pattern than that apparent in the controls, resembling the 'honeycomb' pattern described by *Soderström* (1966) No mitoses were observable until 36 hours after hepatectomy, after that period approximately 3 per cent of the hepatocytes were in different stages of mitosis At 48 and 72 hours the amount of mitotic cells was approximately 1 per cent

One day after the injection of TAA, the hepatocyte nuclei appeared swollen and nucleolar enlargement was noted Fatty vacuolization of the cytoplasm was conspicuous and the cytoplasmic pattern was very distinctly of the 'honeycomb type At 2 days extensive nuclear pyknosis was observed and the liver cells appeared irregular and edgy Marked granulocyte infiltration was also noted At 4 days, the picture very closely resembled that seen after 1 day, although the number of nucleoli was larger and varied between 2 and 5 per cell At 6 days nuclear swollenness and nucleolar enlargement were observable in about 50 per cent of the hepatocytes, the rest appeared to be almost normal Nine days after the injection the liver cells did not differ from those of the control preparations

After 2 days of ANIT treatment hepatocellular changes appeared as dilated intercellular spaces and a pronounced 'honeycomb' pattern Those changes were even more conspicuous after 4 and 6 days At 6 days, moderate, and at 8 days, marked

Fig. 1 Normal hepatocytes. MGG × 1300

Fig. 2 Hepatocytes 3 days after partial hepatectomy. MGG × 1300

Fig 3 Normal hepatocytes Nase × 1300

Fig 4 Hepatocytes 24 hours after partial hepatectomy Nase × 520

Fig 5 Hepatocytes 4 days after the adm n strat on of TAA N ase × 520

Fig 6 Hepatocytes af er 2 days of ANIT treatment N ase × 1300

Fig 3 Normal hepatocytes N ase × 1300

Fig 4 Hepatocytes 24 hours after partial hepatectomy N ase × 520

Fig 7 Normal hepatocytes, SDH × 520

Fig 8 Hepatocytes 48 hours after the administration of TAA, SDH × 520

granulocyte infiltration was remarked *After* 10 and 15 days, bile duct endothelium cells were abundant Nucleolar enlargement was observed after 10 days of treatment but *not* after 15 days

N ase In the control preparations staining for N ase revealed strong enzyme activity in *the bile* canaliculi which appeared as brilliant red bands between and around the hepatocytes (Fig 3) The canalicular borders were smooth and distinct and the general pattern was that of a regular *network*

After hepatectomy the basic canalicular pattern was disturbed by extensive *branching* (Fig 4) Twiggy and hand shaped processes could be seen sprouting *from the* canaliculi in a disorganized manner The *canalicular* borders were knotty and the *canaliculi seemed* tortuous Those changes were visible as early as 20 *hours after hepatectomy* Three days after the *operation* the picture had become *almost normal, although* branching was still *apparent* in some cell groups

One day after the injection of TAA canalicular branching was conspicuous *Signs of* hepatocellular or canalicular damage were *not* discernible At 2 days extensive canalicular damage was noted, canaliculi were to be seen in a few cell groups only while most liver cells stained diffusely red Four days after the injection canalicular branching and *knottiness were again apparent,* while the strong intracellular reaction was absent (Fig 5) At 6 days canalicular branching had diminished and 9 days after the injection the findings were identical with those made in the controls

After 1 day *of* ANIT treatment marked canalicular *dilatation* was apparent The canalicular borders were indistinct and knotty and small canalicular diverticula were occasionally observed At 2 days the changes were even more pronounced (Fig 6) The canalicular borders had completely vanished in some cell groups and enzyme positive coloration was *diffusely* scattered over the cytoplasm After 4 days most cell groups presented no more than a very weak canalicular N ase activity and after 6 and 8 days *most cells* were completely devoid of canalicular activity After 10 and 15 days of *treatment* the reaction seemed rather more marked, and most cell groups possessed a *faintly staining* canalicular network

β gluc Staining for β gluc to some extent *destroyed* cellular morphology, for the most part the preparations were transversed by *thin cracks* which were in some instances also seen to encircle the hepatocyte *nuclei* Hepatocellular enzyme activity was represented by small cytoplasmic granules which *stained deep red*

One day after hepatectomy the enzyme reaction was stronger than in the controls At 2 days *the reaction* was disturbed by fatty vacuolization, to some extent the dye diffusely stained the contents of the vacuoles At 3 days a normal enzyme reaction was observed

After the injection of TAA no changes occurred until the fourth day at which time the strength of the reaction was markedly increased, at 6 days the findings were generally *the same After 9* days the picture did *not differ from* that observable in the controls

During the 6 first days of ANIT treatment there was observed a reaction stronger than normal, but after 8 days most cells stained very weakly and the number of granules was less than in the *controls After 10 and 15* days the findings were identical with those in the controls

SDH Staining for SDH gave a fine granular reaction in the hepatocyte cytoplasm (Fig 7), the granules were dark blue In addition a weak cloudy background coloration of the cytoplasm was noted The granules in some cells seemed to be arranged in coarse irregular rows The hepatocyte nuclei were surrounded by a monolayer ring of flat granules The staining method seemed prone to artifacts as free formazan granules were scattered over the background substance between the liver cells (Figs 7 8)

After hepatectomy the granules were somewhat larger than normal their number was grossly the same as in the controls The perinuclear ring was absent and the distribution

nalicular branching was the main feature in N ase stained preparations, the canalicular pattern very much resembled that seen after partial hepatectomy *Reddy et al* (1969) have reported on hepatocellular proliferation after the administration of 50 mg TAA/kg b w to rats, as determined by the rate of H^3 thymidine incorporation into DNA The present finding of canalicular branching after the administration of TAA might be due to such an effect of the drug After the stage of hepatocellular injury 2 days after drug administration, a normal cytological picture was observable at 4 days in MGG-stained preparations, the enlarged nucleoli suggested hepatocellular proliferation At the same time staining for N-ase revealed canalicular branching The situation at 4 days very probably represents a stage of hepatocellular regeneration after TAA induced liver injury The finding of canalicular branching at that time thus confirms the impression that the phenomenon represents canalicular proliferation

ANIT has been applied to induce intra hepatic cholestasis in experimental animals (*Desmet et al* 1968) The pathogenesis of the cholestasis is unknown, although it is thought that the drug acts by inducing inflammatory lesions in the bile ducts (*Desmet et al* 1968) The finding of bile canalicular dilatation detectable as early as 24 hours after the first dose of ANIT is in accord with the report of Desmet and co workers The abundance of bile duct endothelium cells seen after 10 and 15 days of ANIT administration is attributable to the ANIT induced bile duct proliferation originally reported by Marzanti & Lopez (*Desmet et al* 1968)

The recognition of mitoses in cytological liver specimens is of course an indication of hepatocellular proliferation Even during very rapid liver proliferation mitotic liver cells are observable in relatively small number however, consequently it is concluded that a more useful sign of hepatocellular proliferation seems to be the typical bile canalicular pattern demonstrable by staining for N ase This is the case especially in clinical work where a fine needle aspiration smear does not always contain enough material for a dependable counting of mitoses to be performed.

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of granules was completely irregular The picture was the same after 1, 2, and 3 days

One and 2 days after the injection of TAA the SDH granules were larger than in the controls (Fig 8) At 4 days, the size of the granules was considerably less and a ring of granules surrounded most hepatocyte nuclei After 6 and 9 days the findings were identical with those in the controls

No changes in staining properties were observable in connexion with ANIT treatment until the tenth day At that time the size of the granules was increased, the number of granules was about the same as that in the controls After 15 days of treatment the number of granules was less than normal

Throughout the experiment no differences in staining properties could be observed between the normal and sham controls

DISCUSSION

Partial hepatectomy, as performed by the method of *Higgins & Anderson* (1931) results in the loss of 60–70 per cent of liver tissue By reason of its great regenerative capacity the liver is very suitable for a study of the course of proliferation

Cytoplasmic fatty vacuolization in connexion with liver regeneration after partial hepatectomy has been discussed lately by *Bengmark* (1969) The phenomenon was very efficiently demonstrated in the present study by application of the Oil red O staining method The aetiology of this deposition of fat in the liver cells is not clear the reason is probably to be found in the altered metabolic situation brought about by the liver resection

Bile canalicular dilatation and multiplication have been reported to be demonstrable in histological liver preparations in conjunction with hepatocellular proliferation and various explanations have been suggested for those phenomena (*Bartok & Viragh* 1966) The present findings indicate that bile canalicular multiplication is the result of canalicular proliferation the proliferation process occurs by branching from previously formed canalicular structures Bile canalicular proliferation might be induced by the increased secretory function of individual liver cells brought about by the diminished total liver cell mass The same canalicular pattern is often seen in hepatic cirrhosis of man (*Wasastjerna et al* 1970 b)

The finding of increased β gluc activity of the liver cells after partial hepatectomy is in agreement with the result of the biochemical study of liver proliferation made by *Klockars & Wegelius* (1969) According to their report hepatocellular β gluc activity is increased during the first 2 days after partial hepatectomy following which a decrease of activity takes place

Enlargement of SDH granules accompanies bile canalicular proliferation The phenomenon is probably caused by increased hepatocellular SDH activity during the stage of cell destruction 2 days after the administration of TAA mitochondrial injury appears to be the cause of the increased granular size The staining method is not suitable for quantitative estimation of the activity of SDH as the method seems prone to artifacts extracellular formazan granules were apparent in most preparations stained for SDH

Histochemical alterations of the liver following the administration of TAA have been reported by many authors (*Corcos et al* 1967), the main changes are depression of the activities of various mitochondrial and microsomal enzymes mitochondrial degeneration glycogen depletion and cytoplasmic fatty vacuolization *Gupta* (1956) has reported that a dose of 200 mg TAA/kg b w given intraperitoneally to rats results in the occurrence of centrilobular necroses and the author and others (*Wasastjerna et al* 1970 a) have reported that signs of hepatocellular injury occur within 12 hours on administration of the drug intraperitoneally and in a corresponding dosage to guinea pigs In the present study however no signs of hepatocellular damage *could be observed 24 hours after* the administration of TAA whereas ca

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TIME SEQUENCE STUDY OF NORADRENALINE DEPLETION OF KIDNEY, SPLEEN AND HEART AND OF JUXTAGLOMERULAR DEGRANULATION IN MICE WITH EHRLICH ASCITES CARCINOMA

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Albino mice were injected intraperitoneally with Ehrlich ascites carcinoma. Experimental animals and intact controls were killed at 2 3-day intervals for 16 days after injection Samples were taken from the kidney for histochemical estimation of the noradrenaline content of perivascular sympathetic nerve fibres and for evalution of the juxtaglomerular granularity index Noradrenaline was estimated biochemically in the kidney, heart and spleen We found histochemical signs of noradrenaline depletion that was maximal about 7 days after injection and most pronounced in the smallest arterial blood vessels Noradrenaline levels were normal 12 days after injection, but later were again subnormal The biochemical assay showed a reversible 10 to 30 per cent noradrenaline depletion in the kidney and heart Corresponding findings in the spleen were of doubtful validity because of a considerable increase in spleen weight Different possible causes of these phenomena are discussed

In mice with Ehrlich ascites carcinoma noradrenaline depletion has been demonstrated histochemically in the kidney, heart and spleen and biochemically in the kidney (*Tallqvist & Jansson* 1969, *Tallqvist et al* 1970) A pilot experiment showed that depletion might be reversible, and that depletion was maximum at the time of ascites formation Thus it could be connected with the sodium and water retaining mechanisms, for instance the renin angiotensin system

The object of the present experiments was to find out whether noradrenaline depletion is reversible, and whether it can be correlated with the juxtaglomerular granularity index. According to *Hartroft & Hartroft* (1953) this index can be considered an indicator of the activity of the renin angiotensin system (*Tobian et al* 1959, *Rapp* 1969)

Received 15 i 71

MATERIAL AND METHODS

Among 170 albino mice of both sexes, 106 were injected intraperitoneally with Ehrlich ascites carcinoma (according to *Wager & Rasanen* 1966) and 64 were used as controls The animals were weighed at two-three-day intervals At intervals from 2 to 16 days after injection 6 to 10 experimental animals and an equal number of controls

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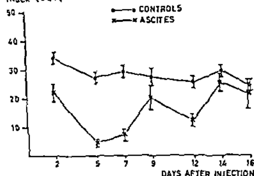


Fig 3 The juxtaglomerular index at various times after intraperitoneal injection of Ehrlich ascites carcinoma. Means + — S E N as in Fig 1

the presumed depletion of noradrenaline was biphasic, with remission at the 12th day There were no or neglible percentages of negative estimations on the fluorescence of the interlobular and arciform arteries The estimations of faint perivascular fluorescence in the various categories of kidney arteries are shown in Fig 2 There was the same biphasic pattern as in Fig 1 The fluorescence was estimated as faint in small percentages of slides in the largest (arciform) arteries and these vessels resembled those in controls at the end of the observation period

The juxtaglomerular granularity indices are shown in Fig 3 The indices in ascitic mice were lower than those in controls exept for the 9th, 14th and 16th days after injection The curve in roughly biphasic An example of low granularity (ascitic mice) and of normal granularity (controls) is offered in Fig 4 and 5

Biochemical investigation The concentrations of noradrenaline in kidney, heart and spleen are shown in Fig 6 The concentrations in ascitic mice were 10 to 30 per cent lower than those in controls for most of the observation period, but reached control values at the end For most of the time the spleens of ascitic mice were clearly heavier than those of the controls (see Fig 7) This might explain why noradrenaline concentra-

Fig 4 Micrograph of the juxtaglomerular apparatus of an ascitic mouse five days after injection of Ehrlich ascites carcinoma. The granularity is decreased Bowie s stain ×1000

were killed with chloroform Histochemical investigation Immediately after killing the animal, part of the right kidney was frozen in propane butane precooled in liquid nitrogen, freezedried and exposed to formaldehyde vapour under standardized conditions For details of the technique, see *Eranko* (1967) and *Corrodi* & *Johnsson* (1967) Pairs of slides were made from each specimen, coded, and examined independently by two of us The blood vessels of the kidney were examined for the specific greenish fluorescence of the perivascular sympathetic varicose fibres The intensity of the fluorescence was estimated subjectively, after viewing ten sections on each slide, as clear (=weak or strong), faint (=weak and occasional) or absent Thus two estimates were made per animal, and in calculating the results each estimation (=slide) was counted as an independent observation Juxtaglomerular granularity index Immediately after killing the animal, part of the right kidney was taken, fixed in Helly's fluid embedded in paraffin, cut and stained with Bowie's stain (*Lynch et al* 1969) The index was determined according to *Hartroft* & *Hartroft* (1953) Biochemical determination of noradrenaline This was carried out as described in our previous paper (*Tallqvist et al* 1970) i e amine extraction according to *Brownlee* & *Spriggs* (1965) and fluorometric assay according to *Laverty* & *Taylor* (1968)

RESULTS

Histochemical investigation The specific fluorescence of adrenergic varicose fibres was

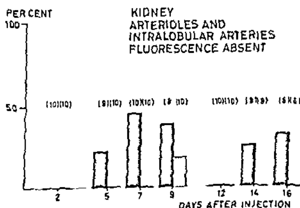
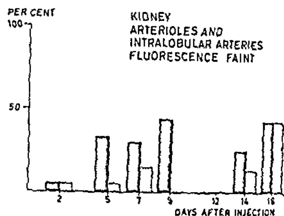
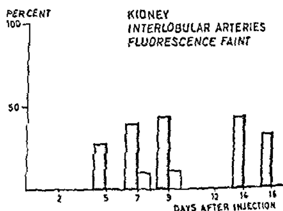


Fig 1 The percentage of slides without specific fluorescence in the arterioles and intralobular arteries of the kidney of ascitic mice (hatched columns) and in intact controls (spotted columns) at various times after intraperitoneal injection of Ehrlich ascites carcinoma Numbers in brackets indicate numbers of animals (N) 100 per cent is equal to 2×N slides





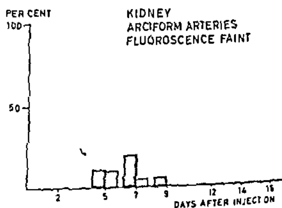


Fig 2 The percentage of slides with faint fluorescence in the blood vessels of the kidney at various times after intraperitoneal injection of Ehrlich ascites carcinoma N as in Fig 1 100 per cent is equal to 2×N slides

estimated as absent in kidney arterioles and intralobular arteries as shown in Fig 1 The fluorescence was like that of controls at the 2nd and 12th days after injection However, there were varying percentages of negative observations 5 to 9 days after injection and at the end of the observation period Thus

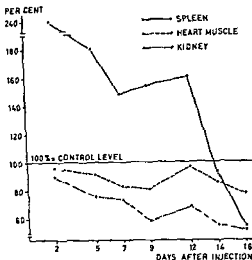


Fig 7 The wet weight of kidney, heart and spleen of ascitic mice expressed in per cent of control values at various times after intraperitoneal injection of Ehrlich ascites carcinoma N as in Fig 1

endogenous level (*Fuxe & Sedvall* 1964, *Anden et al* 1966) The proportionality is linear up to 30–40 per cent, above which a concentration dependent quenching of the fluorescence occurs (*Olson et al* 1968, *Johnsson* 1969)

At every step of our investigation experimental specimens and controls were treated in parallel and blindly We therefore feel that subjective and artefactual biases are excluded as far as possible The occurrence of absent and faint fluorescence in the normal group must be considered as an indicator of the error of method

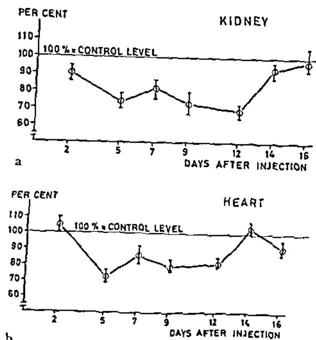
The biochemical assays of noradrenaline in the heart confirms the earlier histochemical finding of depletion in the heart of ascitic mice (*Tallqvist et al* 1970) This underlines the systemic nature of the depletion

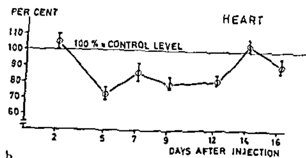
The biochemical assay showed the loss of 10 to 30 per cent of the noradrenaline pool of the heart and kidney Histochemically depletion was most apparent in the smallest vessels and small or negligible in the largest vessels This might be explained by the histochemical findings by *Vogel et al* (1969) in steers with experimental heart failure with oedema They showed noradrenaline depletion of terminal varicose fibres in close association with cardiac muscle cells Little or no change occurred in preterminal fibres or in terminal fibres in connective tissue septa or around blood vessels The relevancy of this explanation depends on the similarity between heart failure with oedema and ascites, and on the assumption that small arteries contain more terminal fibres than do larger arteries

Both biochemical and histochemical results showed that the noradrenaline depletion in ascitic mice was reversible Its maximum was about the 7th day after injection We cannot decide whether the depletion was a reversible response to a linearily increasing stress factor, i e the growth or the tumour, or, whether the depletion, being maximal in the middle of the period of ascites formation, was correlated with sodium and water retention After severe thermal injury, stress, hypoxia or haemorrhagic hypotension noradrenaline depletion was shown biochemically in the adrenal medulla and sympathetic nerves of human subjects (*Goodall & Haynes* 1960, *Goodall & Moncrief* 1965) and rats (*Coca & Glaviano* 1968), in the brain, heart, liver and spleen of rabbits, rats and dogs (*Hökfelt* 1951, *Coleman & Glaviano* 1963, *Hashimoto* 1965, *Hift et al* 1965, *Bhagat* 1969, *DeSchaepdryver et al* 1969, *Fatman* 1969) Noradrenaline depletion was shown biochemically in the heart of rats after adrenal demedullation and hypophysectomy (*Hokfelt* 1951, *Borchard & Vogt* 1970) but not after adrenalectomy (*Bhagat* 1969, *Borchard & Vogt* 1970) ACTH had the opposite effect (*Hökfelt* 1951) The noradrenaline depletion we found might therefore be a reversible response to the stress of the growing tumour

A correlation with sodium and water retaining mechanisms is favoured by several facts Noradrenaline depletion was shown in terminal sympathetic nerve fibres of the heart in association with experimental heart failure and oedema in steers (*Vogel et al* 1969)

Fig 5 Micrograph of the juxtaglomerular apparatus of a control mouse at the fifth day The granularity is normal Bowie's stain ×1000





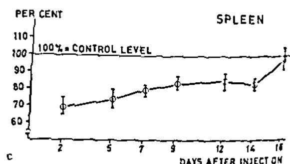


Fig 6 Noradrenaline concentration is the kidney, heart and spleen of ascitic mice expressed in per cent of control values + — S E at various times after intraperitoneal injection of Ehrlich ascites carcinoma N as in Fig 1

tions in the spleen were 70 to 97 per cent of control values As the increase in weight of the spleen probably depends on hyperaemia DNA would have been a better denominator than wet weight for the concentration assays.

DISCUSSION

Subjective estimation of formaldehyde induced fluorescence appears to be a rather dependable measure of noradrenaline content of the structure examined The lower limit of detectability is 5 to 10 per cent of the

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and rabbits (*Fizel & Fizelova* 1969), and in human subjects with heart failure and oedema (*Chidsey et al* 1963, *Chidsey et al* 1965, *Kramer et al* 1968)

The juxtaglomerular granularity index can be considered a measure of the plasma renin activity, degranulation indicating repressed and hypergranulation increased activity (*Hartroft & Hartroft 1953, Hartroft et al* 1959, *Tobian et al* 1959, *Hartroft* 1963, *Bozovic et al* 1968, *Cannon et al* 1968, *Maxwell* 1969, *Rapp* 1969) Even in acute experiments, stimulation of the juxtaglomerular apparatus (clipping of the renal artery, administration of hypotensive drugs or ACTH) resulted in increased juxtaglomerular granularity within 30 minutes to 2 hours (*Garber et al* 1959, *Marks et al* 1960, *Simpson* 1965, *Koletski et al* 1967) Only *Miller & Hartroft* (1961) reported that ingestion of a sodium deficient meal was followed by transient degranulation of the juxtaglomerular cells "within the first few hours" After this, granulation progressively increased with continuation of the dietary regimen Therefore we consider the juxtaglomerular degranulation we found in ascitic mice to be a sign of depressed plasma renin activity

Conditions associated with ascites and oedema cause hypergranulation of juxtaglomerular cells This is seen in dogs whose thoracic vena cava has been constricted (*Davis et al* 1962) and in rats with ascites following aminonucleoside nephrosis (*Tobian et al* 1962) In man hypergranulation is seen in heart disease with oedema and in liver disease with ascites (*Reeves et al* 1962, *Hartroft & Hartroft* 1961) This state is also characterized by high plasma reninangioten sin levels (*Veyrat et al* 1968, *Haber* 1969) Thus the juxtaglomerular degranulation we found was rather unexpected

We found that noradrenaline depletion and juxtaglomerular degranulation ran roughly parallel in time This might of course be coincidence, but they might be correlated As a hypothesis we suggest that the depletion of noradrenaline might prevent efferent impulses from the sympathetic nervous system from reaching the juxtaglomerular apparatus (*Wågermark et al* 1968, *Ljungqvist & Wågermark* 1970) and thereby inhibiting the sympathetic stimulus to renin production, the importance of which has been shown by many authors (*Vander* 1965, *Bunag et al* 1966, *Bozovic & Castenfors* 1967, *Cohen et al* 1967, *Michelakis et al* 1969, *Werning & Siegenthaler* 1969, *Winer et al* 1969) This interpretation is supported by the fact that renal denervation blocked the increase in plasma renin activity resulting from sodium depletion (*Mogil et al* 1970)

In this study the loss of noradrenaline from sympathetic nerve fibres was biphasic, with normal values on the 12th day after injection This might imply that, by the 12th day, the initial depletion was compensated for by accelerated synthesis, but by the end of the observation period compensation was inadequate and depletion was again manifest (=histochemically visible) This interpretation is supported by the fact that hypoxia resulted in biphasic catecholamine depletion in the adrenals, with signs of accelerated synthesis following the initial depletion (*Steinsland et al* 1970) This problem is the object of current experiments

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DOPAMINE BINDING IN A TRANSPLANTABLE ISLET CELL TUMOUR OF GOLDEN HAMSTER

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Differential centrifugation of homogenates from a transplantable islet cell tumour of the golden hamster revealed that most of the 5 hydroxytryptamine and histamine, but, surprisingly, only a small part of the dopamine, is bound to particles Of the dopamine that could not be spun down by centrifugation, about one third was included in a non dialysable fraction that could be precipitated by addition of ammonium sulphate and, moreover, the dopamine runs together with the proteins on a Sephadex G 25 column These observations suggest that part of the tumour dopamine is not particle bound, but is associated with protein

Biogenic monoamines, especially dopamine, are stored in the pancreatic islet cells of many mammalian species (c f *Cegrell* 1968) The golden hamster is one of the few species where no monoamines have been found in the adult endocrine pancreas On the other hand, dopa, dopamine, and 5 hydroxytryptamine have recently been reported (*Cegrell et al* 1969 a) to occur in the transplantable islet cell tumour of golden hamster that was originally described by *Kirkman* (1962) It has also been demonstrated that tyrosine hydroxylase and dopa decarboxylase, the two enzymes catalyzing the synthesis of dopamine from tyrosine are present in the tumour (*Axelsson et al* 1970, *Cegrell et al* 1969 b) Thus it seems that the tumour cells can store monoamines and synthesize at least dopamine

The dopamine storing tumour cells could be expected to contain numerous storage granules, not least because this amine seems to be stored in granules in normal B cells (*Cegrell* & *Falck*, unpublished) Electron microscopic studies, however, have revealed that the tumour cells contain only scarce amounts of granules and that many cells seem to be agranular (*Simar et al* 1968, *Falkmer et al* 1969) These chemical and microscopical observations prompted the present study on dopamine binding in hamster insuloma

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MATERIAL AND METHODS

Islet cell tumour tissue was transplanted subcutaneously to golden hamsters and allowed to grow for four to eight weeks, at which time the tumours weighed 4 8 g The tumours, which showed no signs of necrosis at dissection, were cut in pieces and gently homogenized with a Potter Elvehjem homogenizer in 0 32 M sucrose solution (5 ml per g tissue) containing 10 μM $CaCl_2$ To remove unbroken cells, cell debris, and large cell organelles, the tissue was first centrifuged at 900×g for 10 min The supernatant was then centrifuged at 20,000

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it seems reasonable to suppose that this fraction of tumour dopamine is bound to protein. With this assumption as a working hypothesis, the current studies are aimed at checking in more detail the specificity of such a binding.

The observation that the precipitates contained only about 30 per cent of dopamine prompted us to investigate whether the ammonium sulphate could rupture a possibly existent dopamine protein complex. To check this the high speed supernatant was dialysed immediately after centrifugation without adding ammonium sulphate (2 experiments). As the amount of non dialysable dopamine remained the same as that obtained after precipitation, the same dopamine-protein complex could well be present in the two experiments. An unspecific adsorption of dopamine to a protein in a way known to occur between many drugs and proteins should also be excluded. Dopamine was therefore added to albumin and blood plasma. However, after precipitation with ammonium sulphate, no non dialysable dopamine could be detected. Nor were any non-dialysable catecholamines found in the high speed supernatants obtained from the cow adrenal medulla or the caudate nucleus and pancreas of the pig, two tissues known to be rich in dopamine (for pancreatic dopamine see Cegrell 1968).

As the islet cell tumour contains mast cells, a dopamine heparin complex precipitable by ammonium sulphate had to be excluded. A high speed supernatant was therefore prepared from cow liver capsule, a tissue rich in mast cells carrying high amounts of dopamine (Falck et al 1964). However no dopamine was found in the precipitate after addition of ammonium sulphate.

The experiments have demonstrated particle-bound dopamine in the islet cell tumour. The distribution of 5 hydroxytryptamine and histamine in the hamster insuloma differs considerably from that of dopamine, three quarters being found in the sediments, which means that these amines, to a greater extent than dopamine, are bound to particles (see Table 1). In contrast to dopamine, neither of these amines seems to be bound to large molecules since they did not occur in the precipitates produced by adding ammonium sulphate to the high speed supernatant.

The experiments have thus shown that, apart from the "unbound" dopamine that could be expected to occur in insuloma as in other tissues, part of the tumour dopamine is associated with the particular fraction, whereas a significant amount seems to be bound to a large molecule, possibly a protein. This is probably not an artifact because attempts to provoke an unspecific binding of dopamine to non dialysable molecules in other tissues failed. The finding can at least partly explain why the tumour tissue can carry high amounts of dopamine despite the cells containing only few granules.

This work was supported by grants from the *Swedish Cancer Society* (67-111) and was carried out within a research organization sponsored by the *Swedish Medical Research Council* (Projects No B71 14X 56-07A and B71 14X-712-06A)

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×g for 20 min and the resultant supernatant at 100,000×g for 60 min The last supernatant and sediment obtained are, in the following, called 'high speed supernatant' and 'high speed sediment' All operations were performed at +4°C

Determinations were made of the dopamine, 5 hydroxytryptamine, and histamine contents of the three sediments and high speed supernatant (*Bertler et al* 1958a, b, *Bertler* 1961, *Kurahashi* & *Fujiwara* 1969) One ml of the high speed supernatant was used for the dopamine determination, ammonium sulphate (pH 7 0) being added to the rest to 40 per cent saturation In one series of experiments, the precipitates obtained were dissolved in 7 ml 0 04 M sodium phosphate buffer (pH 7 0) and then dialysed against water for 16 hours at +4°C The content of dopamine in the non dialysable material was determined as mentioned above, and the identity of the amine was further established by paper chromatography in phenol 0 1 N HCl (9 1) according to *Bertler et al* (1958a) As controls, we used identically treated tissues known to contain high amounts of dopamine namely pig caudate nucleus pig pancreas, and cow liver capsule, and one tissue containing high amounts of adrenaline and noradrenaline, i e adrenal medulla from the cow In another series of experiments the ammonium sulphate precipitates were dissolved in 1 5 ml 0 01 M phosphate buffer (pH 7 0) and made isotonic with sodium chloride and then passed through a column (30× 1 5 cm) containing Sephadex G 25 The elution was performed with about 40 ml of the phosphate buffer and the effluent gathered in 2 ml fractions The dopamine content of each fraction was determined and the protein content was estimated spectrophotometrically using the absorption at 280 nm

In two experiments the high speed supernatant was centrifuged for 2 hours at a still higher speed (rmax 361 300×g) using a Spinco L2 65B preparative ultracentrifuge (rotor 60 Ti 60 000 rpm) and dopamine was determined in the sediment

To investigate the specificity of a possible dopamine binding to proteins in insuloma (see Results) the following control experiments were performed Ten μg dopamine was added to 10 ml human blood plasma (2 experiments) and to 300 mg human serum albumin in 5 ml of the phosphate buffer (2 experiments) and the samples were allowed to stand for 2 hours at 37°C To the incubation mixtures ammonium sulphate was added to 40 per cent saturation and the precipitates dialysed against water overnight The dopamine content of the non dialysable fraction was determined

RESULTS AND DISCUSSION

As Table I indicates, differential centrifugation of the insuloma showed that dopamine

TABLE 1 *Distribution in Per Cent ± S.D of Dopamine (DA) 5 Hydroxytryptamine (5 HT) and Histamine in Sediments and Supernatant after Differential Centrifugation of Insuloma Figures in Brackets Indicate number of Experiments*

Amine	20 000×g sediment	High speed sediment	High speed supernatant
DA	16± 3(9)	15± 5(9)	69± 6(9)
5 HT	52±12(3)	25± 6(3)	23± 6(3)
Histamine	39 , 32	49 , 53	12 15

occurred mainly in the high speed supernatant, although significant amounts of dopamine were present also in the sediments spun down at 20,000×g and 100,000×g The presence of dopamine in these sediments demonstrates that part of the dopamine is stored in particles To test whether the dopamine of the high speed supernatant was bound to small particles not sedimented at 100 000×g the high speed supernatant was in two experiments centrifuged at 360,000×g for 2 hours No dopamine was found in the sediments thus obtained

Simar et al (1968) and *Falkmer et al* (1969) found only scarce amounts of tumour cell granules in electronmicroscopic analyses In our preliminary studies granular structures of similar electron density and size have been found in both the 20 000×g and high speed sediments Further investigations will reveal whether these structures represent the particular storage site for dopamine in the islet cell tumour Recently *Orci* (personal communication 1970) and *Creutzfeldt* (personal communication 1970) have reported that in these tumour cells there are granules similar to those of the adrenal medulla

After addition of ammoniumsulphate to the insuloma high speed supernatant and subsequent dialyses of the precipitate, 30±5 per cent of the dopamine was found in the non dialysable fraction (10 experiments) The identity of the amine was further established by paper chromatography When such a precipitate was passed through a Sephadex column (9 experiments) dopamine was found only in the protein containing fractions Thus

it seems reasonable to suppose that this fraction of tumour dopamine is bound to protein With this assumption as a working hypothesis, the current studies are aimed at checking in more detail the specificity of such a binding

The observation that the precipitates contained only about 30 per cent of dopamine prompted us to investigate whether the ammonium sulphate could rupture a possibly existent dopamine protein complex To check this the high speed supernatant was dialysed immediately after centrifugation without adding ammonium sulphate (2 experiments) As the amount of non-dialysable dopamine remained the same as that obtained after precipitation, the same dopamine protein complex could well be present in the two experiments An unspecific adsorption of dopamine to a protein in a way known to occur between many drugs and proteins should also be excluded Dopamine was therefore added to albumin and blood plasma. However, after precipitation with ammonium sulphate, no non dialysable dopamine could be detected Nor were any non dialysable catecholamines found in the high speed supernatants obtained from the cow adrenal medulla or the caudate nucleus and pancreas of the pig, two tissues known to be rich in dopamine (for pancreatic dopamine see Cegrell 1968)

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CYTOTOXIC ANTIBODIES AND ACCELLERATED REJECTION OF THE TRANSPLANTED RABBIT HEART THE EFFECT OF PRESENSITIZATION

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In a group of 20 unmodified rabbit recipients receiving a cardiac allograft 7 rabbits formed lymphocytotoxic antibodies The histological features of rejection were identical in all cases, independent of antibody formation Thirteen rabbits were presensitized against the prospective heart donor by means of skin grafts and spleen cell injections, and the majority of these recipients were transplanted in the presence of donorreactive cytotoxic antibodies Accellerated rejection took place in every recipient but the rapidity of the reaction and the histological picture did not correlate well with the presence and potency of preexisting antibodies Histological findings varied considerably including infiltration of mononuclear cells and polymorphonuclear leucocytes, extensive oedema and interstitial haemorrhage, and early degeneration of myofibres Only one case of 'hyperacute" clinical rejection was observed in which the transplant was rejected within 2 1/2 hrs Heavy deposits of gammaglobulins were demonstrated by the fluorescent antibody method to be localized in the vessel walls of this transplant

Investigations on renal allograft rejection by rabbits previously challenged with skin transplants (9 10) or kidney transplants (8, 10) have shown that hyperacute rejection eventually take place, within the species, and that *transplantation antibodies* may play an important role in the pathophysiological events during rejection It has been proposed from allografting experiments carried out on rats, (14) that hearts either elicit a more vigorous immune reaction than kidney or, that transplanted hearts are more susceptible to immune attack than renal transplants.

The present investigation was undertaken in rabbits to demonstrate if circulating cytotoxic antibodies are formed during cardiac allograft rejection and to investigate the role of presensitization for the rejection of heart transplants

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MATERIAL AND METHODS

New Zealand White, Brown Lop-eared, Black Alaska rabbits, and interstrain crosses weighing 2-4 kg were used as recipients Donors were unrelated young rabbits aged 45-60 days and weighing 0 5-1 2 kg Grafts were placed in the neck using the technique previously described (4), and removed when contractility and electrical activity

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TABLE 2 *Summary of Serological Functional and Morphological Results in Presensitized Recipients*

Rabbit recipient reference number	Presensitisation		Cytotoxic antibody potency	Graft function		Histology			Immunofluorescence			
	Skin	Spleen		Survival days	Removed days	Mono	Poly	Oed	γ cell	Sarc	Vess	Myo
1 S	0	+	—	4½	4½	+ +	+ +	+	+ +	+	+	+
2 S	0	+	+ +	4	4	+ + +	—	—	—	+ +	—	+ +
3 S	0	+	—	4	4	+ +	+	—	+	+	—	+
4 S	0	+	+ +	1	1	+	+ +	+ +	—	+ + +	+	+
5 S	0	+	+	–	3	+ +	+	—	—	+	—	+
6 S	1	+	+	4	4	+ + +	+	+	—	+	+	+ + +
7 S	1	+	+	1½	1½	+ + +	—	+	+	+ +	+	+
8 S	1	+	+ +	2½ hours	2½ hours	+	+	+ + +	—	+	+ + +	+
9 S	2	+	—	4	4	+	+ +	+	+ + +	+	+ +	+
10 S	2	0	+	5	5	+ +	+	—	—	—	+	+
11 S	6	0	+	4	4	+	+ +	+ +	+	+	—	+
12 S	8	0	+ + +	–	1	+	+ + +	+	—	+ +	+	+
13 S	8	0	+ +	2½	2½	+	+ +	+ + +	+	+	—	+

For abbreviations see text Table 1

were lost No immunosuppressive therapy was given

Sensitization

Full thickness skin grafts measuring 6 cm² were cut from the lateral abdominal wall of the prospective heart donor and were fitted into a *suprapannicular graft bed on the recipient's chest* behind the interscapular region and kept in place by eight silk sutures Cytotoxic tests were performed 14–20 days later and if antibodies were absent, a new skin transplant was made Hemisplenectomies were performed on selected donor animals The spleen was cut into small pieces with scissors, pressed through a fine wire mesh and suspended in Hank's balanced salt solution Ten ml of a suspension containing 2×10^7 nucleated spleen cells per ml were injected subcutaneously and intramuscularly into the prospective recipient

Serological Investigation

Serum samples were obtained from the marginal ear vein of the recipient at varying intervals during sensitization, and during and following heart transplantation Serum was stored in small aliquots at −25°C until investigated Recipient serum was tested for cytotoxic antibodies against lymphocytes from donors of skin, spleen and hearts. Serum collected after transplantation (donor dead) was tested against a randomly selected rabbit cell panel from at least 5 animals Cytotoxic tests were performed using microtechniques

The lymphocyte suspensions were prepared from heparinized blood using a differential centrifugation technique as a modification of the method described by *Boyum* (1)

Fresh pooled normal rabbit serum was used as the source of complement Serum and cells were mixed under oil and incubated for 2 hours at

TABLE 1 *Summary of Functional, Serological and Morphological Results in Unsensitized Recipients*

Rabbit recipient reference number	Graft survival days	Cytotoxic antibody following rejection	Histology			Immunofluorescence			
			Mono	Poly	Oed	γ cell	Sarc	Vess	My
1	10	—	+ + +	—	—	+ +	+	—	+
2	6	—	+ +	—	+	+	+		+
3	11	—	+ +	—	+	+	—		+
4	9	—	+ + +	—	+	+	—	—	+
5	11	+ + +	+ + +	—	+	+	+	+	+
6	7	—	+ +	—	+	—	+	—	+
7	12	—	+ + +	—	—	+ +	+		+
8	8	+	+ + +	—	—	—	—		+
9	9,5	+ +	+ + +	+	+	+	+		+
10	9,5	+	+	—	+ +	—	+	—	+
11	16	+	+ +	+	—	—	+	+	+
12	13 5	+ + +	+ + +	—	+	+	+	+	+
13	13	—	+ +	—	+	—		—	+
14	9	—	+ +	—	—	+	+	—	+
15	7	—	+ + +	—	—	—	+ +		+
16	9,5	+	+ + +	-	+	+	+		+
17	8	—	+ +	+	+ +	—	+		+
18	10	—	+ + +	—	+	—	+		+
19	15	—	+ + +	+	+	+	+ +		+
20	5,5	—	+ + +	—	+		+		+ +

Cytotoxic antibody potency + = 0–25 % killed cells,
+ + = 25–50 %, + + + = 50–75 %
Mono = Mononuclear cell infiltration
Poly = Accumulation of polomorphonuclear leucocytes (photo A)
Oed = Interstitiel oedema and haemorrhage
γ cell = Cells containing cytoplasmatic immunoglobulin (photo C)
Sarc = Sarcolemma b
Vessel = Localization (
Myo = Intracellular

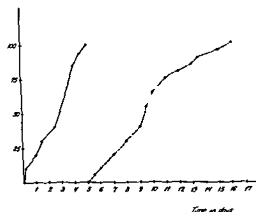


Fig 2 Rejection of cardiac allografts in rabbits
O———O Presensitized recipients
O- -O Unsensitized recipients

lining smaller blood vessels as seen in the majority of rabbits (A) Intense leucocyte reaction was also present in the areas of early necrosis In rabbit No 8 S the graft was hyperacutely rejected clinically and histologically it showed a moderate accumulation of polymorphonuclear neutrophils and massive interstitial oedema and haemorrhage

Immunofluorescence

The pattern of γglobulin localization in the transplants was the same for all unsensitized animals and included spreckled disrupted heavy intracellular deposits in degenerated myofibres and a discreet lining of the sarcolemma Only very few infiltrating cells contained cytoplasmatic γglobulins

γglobulins could be found in the accellerated transplants in the same pattern as mentioned above (Ph B & C) but considerably more pronounced variations within the same graft existed It was furthermore observed that γglobulins were bound to intima and incorporated into the media and adventitia of some larger vessels This picture was very marked in rabbit No 8 S in which case the deposits were present in almost every greater vessel in the rejected transplant (Photo D) In the graft of rabbit No 9 S cellular infil

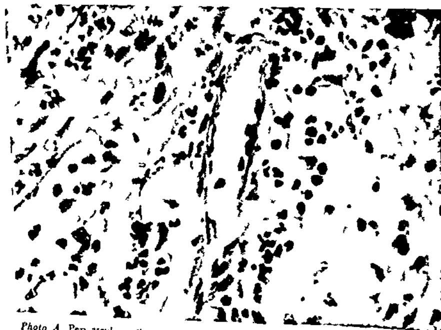


Photo A Perivascular cell infiltrate containing several polymorphonuclear neutrophils Rabbit no 11 S (haematoxylin and eosin × 400)

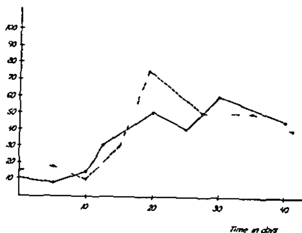


Fig 1 Appearance of cytotoxic antibodies in heart allotransplanted rabbits (unsensitized)

O ——— O Rejection complete on day 11 (rabbit no 5)

O – – O Rejection complete on day 13 (rabbit no 12)

37°C Viability of the cells was determined by trypan blue exclusion

Serum was considered cytotox positive if the viability of the lymphocytes decreased more than 25 per cent following incubation

Preparation

The allografted hearts were removed when contractility was lost and representative tissue was fixed in ethanol 80 per cent and Zenkers formol for paraffin embedding Sections were stained with haematoxylin and eosin methyl green and pyronine and Lendrum s fibrin method

Frozen tissue was cut and stained for immunofluorescence microscopy as described previously (5)

RESULTS

The results are summarized in Tables 1 and 2 and Fig 2 Seven of the 20 rabbit recipients that were not preimmunized formed lymphocytotoxic antibodies during cardiac allograft rejection The antibodies appeared about 10 to 14 days after transplantation and their potency increased during the following three to four weeks (Fig 1) All cytotoxins detected possessed reactivity against more than one of the lymphocyte panel donors, as a rule positive sera reacted with cells from approximately half the number of lymphocyte samples used in the panel

Significant increase in titre at the day of operative removal of the rejected transplants could not be demonstrated

The histological features of rejection did not differ whether transplantation antibodies were absent or present during and following rejection All transplants were rejected acutely (6–16 days) and showed typical mononuclear cell infiltrations as detailed in an earlier paper (5) Survival times of the transplants in recipients with cytotoxic antibodies did not differ significantly from those of the group without these antibodies and the clinical features were identical

The Effect of Sensitization

Cytotoxic antibodies against donor leucocytes were not common following a single skin transplant, when recipient serum was investigated 14 days after transplantations, at a time when rejection of the skin was evident Successive skin grafts were performed until cytotoxins were definitely formed

Injection of spleen cells and in some cases one skin transplant applied 2 weeks later could provoke the formation of lymphocytotoxins in most instances

The rejection process was accellerated in all the presensitized animals independently of presence or absence of cytotoxic antibodies against donor lymphocytes, animals that rejected their transplants very rapidly (2½–36 hrs) had preformed antibodies High potency preformed antibodies could on the contrary not guarantee clinical hyperacute rejection

Microscopic Examination

The histological changes in accellerated rejection were characterized in most instances by an early mononuclear cell infiltrate including a very pronounced number of pyroninophilic cells At final rejection the total infiltrate was usually less marked than that of the first graft, whereas oedema and interstitial haemorrhage were more pronounced Additional histological finding during accellerated rejection was polymorphonuclear leucocytes accumulating around and

trate containing numerous cells with cytoplasmatic γglobulin was evident (Photo C)

Sera in which cytotoxic antibodies to rabbit lymphocytes were detected following rejection were tested for *in vitro* heart reactive antibodies by incubation on cryostat section of rabbit hearts *Immunoglobulin* was found by indirect immunofluorescence to fix to the sarcolemma of ome but not all *normal* rabbit hearts (ph... F)

DISCUSSION

The present studies clearly indicated that an accelleration of the allograft immune response against a heart transplant took place following prior sensitization with donor skin *transplants and spleen cell injection In con* trast to the rather uniform histological alter ations observed in the first set cardiac graft, the histology of accellerated rejection following preimmunization revealed different kinds of reactions varying from case to case and also sometimes within the same transplant This seems to indicate that the process of accellerated rejection reflects that different *immunological mechanisms are working*, and

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Photo B Immunofluorescent staining of myocardium rejected by rabbit no 4 S demonstrating strong sarcolemmal binding (+ + +) *of immuno* globulin G (FITC conjugated goat antirabbit IgG × 250)

Photo C Mononuclear cells exhibiting cytoplasmatic fluorescence Rabbit *no* 1 *S* (FITC *con* jugated horse antirabbit IgG IgA × 400)

Photo D Fluorescent micrograph of *an artery in* the hyperacutely rejected transplant in rabbit No 8 S Gammaglobulin is distributed *in the intima* and media of the vessel wall (FITC conjugated horse anti rabbit IgG IgA × 400)

Photo E Immunofluorescent staining of normal rabbit myocardium incubated with serum from rabbit No 5 S on the 20th day following rejection Sarcolemmal staining is evident demonstrating the presence of circulating heart reactive antibody FITC conjugated swine anti rabbit IgG-IgM × 400

that these sometimes act simultaneously *Clinical* '*hyperacute*' *rejection* (rejection within a few hours) only took place in one donor/recipient combination despite heavy presensitization in many recipients, and it must be concluded that the rabbit heart is less susceptible to the 'hyperacute' clinical rejection course than the rabbit renal allograft (8, 9, 10)

The role of pre-existing donor reactive lymphocytotoxic *antibodies in the various* tissue reactions observed remains obscure, as no exact correlation existed between the presence or the potency of these antibodies and the rejection time and histological picture It seems most reasonable to assume that the serological method of lymphocytotoxicity only *detects some levels of and some special type* of antibodies present following sensitization with allogeneic antigens, and that other forms of humoral antibodies might play significant roles for the rapidity and histological features of accellerated rejection Thus *Klassen* & *Milgrom* (8) have shown in rabbit kidney transplantation experiments that transplantation antibodies detectable by means of a *mixed agglutination test with rabbit cell cul* tures correlated reasonably well with histological evidence of hyperacute rejection This *in vitro* test obviously reflects the *in vivo* serum bound reactivity better than that of cytotoxicity as used in a later study by *Lund* & *Ahrons* (9) on the same subject

In *man*, hyperacute rejection *has* been observed too in the absence of a positive *cytotoxicity crossmatch* (*2, 13*) An immune adherence crossmatch technique was proposed as a more reliable assay system for the prevention of this catastrophic clinical course of a renal transplant

The participation of serum antibody *in* graft destruction has been clearly indicated in earlier studies on *human renal and cardiac* allograft rejection (6, 7, 13, 15, 16 17) In *volvement of cellbound antibody* has not been entirely excluded in these situations, but the importance of circulating factors was suggested by the speed of and the histological features of the '*hyperacute*' reactions in


B


C


D


E

16 *Williams, G M, Hume, D M, Hudson, R P, Morris, P J, Kano, K* & *Milgrom F* 'Hyperacute renalhomograft rejection in man N Eng J Med *279* 611 618, 1968

17 *Williams, G M, Hume, D M, Kano, K* & *Milgrom, F* Transplantation antibodies in human recipients of renal homografts J Amer Med Ass *204* 119–122, 1968

cases with the presence of donor specific serum antibodies

The way in which humoral antibodies contribute to the destruction of transplants is unclear It is suggested that one mode is that antibodies react with antigens of the vascular endothelium and that this reaction may increase vascular permeability and activate the plasma complement system, resulting in the attraction of polymorphonuclear neutrophils and platelets that are ultimately responsible for tissue damage (11)

The vascular lesions with bound antibodies and surrounding granulocytes and the result of increased vascular permeability found in the present study tend to substantiate this hypothesis as does the work of *Najarian & Perper* (12) These workers observed that hyperacute rejection of an allotransplanted kidney could be induced by transfer of serum from allogeneically sensitized animals and showed a localization of globulin in the vascular walls similar to that observed in the present study

Despite the presence of sarcolemma bound antibodies in the cardiac grafts at the time of acute rejection and the finding of *in vitro* heart reactive antibodies in serum following rejection, the role of these in the rejection process still remains speculative Heart reactive antibodies exhibiting analogous fluorescent staining properties have been found in human heart transplant recipients and in serum of patients with rheumatic fever (3)

In conclusion, the results of the experiments which were designed primarily to study second-set rejection advanced the hypothesis that second-set heart transplant rejection is mediated by several participating mechanisms some of which act also in first-set rejections The exact chain of events and combination of morphological features in cases of second-set rejections vary and cannot be accurately predicted from the preimmunization regime or from information on the presence of preformed donor reactive cytotoxic antibodies

The author wish to acknowledge the expert technical assistance of miss *Inny Bang* and miss *Vibeke Barbara Hansen*

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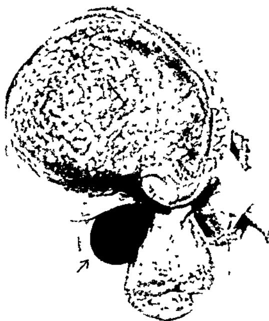


Fig 1 Liver from normal animal (left) and steatotic liver of cholesterol cholic acid fed animal (right) Note distended gallbladder as compared with normal (see arrows) × 3,5

weighed and together with gallbladder, lungs, heart spleen, lymph nodes kidneys adrenals, … r cent … s were … and van Gieson's stain Sections of liver and spleen were also stained for haemosiderin with Turnbull s blue method Frozen sections of liver lungs, heart, spleen, lymph nodes kidneys and adrenals were stained with Scharlach Rot and Sudan Black B and also analysed with the polarizing microscope In addition, sections of the thyroid gland were stained with periodic acid Shiff (PAS) and sections of the pituitary gland with trichrome periodic acid Shiff according to *Pearse* (10) Serum cholesterol levels were determined according to a modified Liebermann Burchard reaction

Experiment 2

One hundred gerbils of both sexes and with a mean weight of 70 g were divided strictly at random into two groups, one of 20 and one of 80 animals. The animals had been fed with normal chow pre-experimentally All animals of the group of twenty were sacrificed after one month on chow They served as controls At autopsy the gallbladder was removed and bile was collected for analysis As the bile samples were small, pooling was necessary As a rule 2–4 animals contributed to each sample 0 050 ml of bile was extracted and hydrolyzed according to the method described by *Wollenweber, Kottke* & *Owen* (16) The free bile acids were separated by thin layer chromatography according to the same method with minor modifications The spots were quantified by means of a Vitatron Densitometer U.F D[2] For further details and for a discussion of the precision and accuracy of this method the reader is referred to *Juul* & *van der Linden* (9). The cholesterol concentration of the bile was determined after extraction according to a modified Liebermann Burchard reaction The lipids were extracted with a mixture of chloroform methanol (2 1) The extract was filtered and evaporated to near dryness The residue was redissolved in petrolether and aliquots were used for determination of lipid phosphorus according to the method of *Chen, Toribara* & *Warner* (5) The phospholipid content was calculated by multiplying the value for lipid phosphorus by a factor of 25 Liver and spleen were weighed and histological examinations were performed as described in Exp 1 Finally blood samples were collected and serum cholesterol levels were determined as previously described

Eighty animals constituted the experimental

[2] Vitatron Dieren, Holland

Acta path microbiol scand Section A 79, 476–486, 1971

REACTION OF THE MONGOLIAN GERBIL TO A CHOLESTEROL-CHOLIC ACID-CONTAINING GALLSTONE INDUCING DIET

Frank Bergman and Willem van der Linden

The Department of Pathology I and the Department of Surgery, University of Umeå, Umeå, Sweden

Mongolian gerbils fed chow supplemented with 1 per cent cholesterol and 0 5 per cent cholic acid, developed steatosis of the liver, accumulation of lipids in the reticuloendothelial system, high serum cholesterol levels and accentuated hypoplastic changes of the thyroid gland In the bile, a marked rise of the cholesterol concentration, a slight rise of the cholic- and deoxycholic and a decrease of the chenodeoxycholic acid concentration was observed After 4 months on the diet, cholesterol gallstones were found in the distended gallbladders Discontinuance of the diet showed the gerbil to be rather unable to free itself from excess cholesterol with a very slow and incomplete return to normal of liver histology In this respect the gerbil differs from the mouse but also a number of similarities in the reaction of these two species to the diet were noted

Comparative studies of gallstone formation in different animals may not only supply valuable information about the conditions which should be fulfilled for gallstone formation to occur They may also help us in selecting certain species as most suitable for further investigations This was the reason why we studied gallstone formation in yet another species, the mongolian gerbil

In the course of our experiments, we observed gallstones in the gerbil after prolonged administration of a cholesterol cholic acid supplemented diet As we in earlier experiments (2) had found that different species react very differently to this diet, we tried to elucidate this kind of gallstone formation by studying the biochemical and morphological changes—their development and regression—in the gerbil

MATERIAL AND METHODS

Experiment I

Thirty eight mongolian gerbils of both sexes with a mean weight of 60 g were randomly divided into two groups All animals were individually caged and had free access to food and water One group, comprising 30 animals, were fed with chow[1] to which 1 per cent cholesterol and 0 5 per cent cholic acid had been added The other 8 served as controls After 4 months the surviving animals were sacrificed after they had been weighed The gallbladder was incised, permitting the contents to run out on blotting paper for subsequent study under a binocular stereomicroscope The liver was

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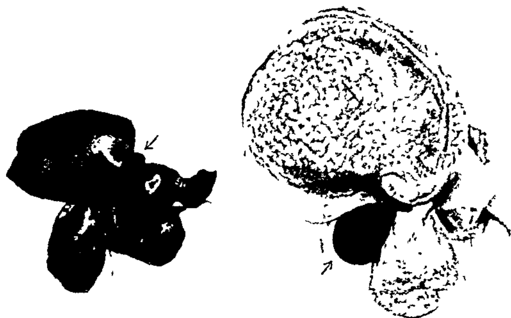


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weighed and together with gallbladder, lungs, heart spleen lymph nodes, kidneys, adrenals, thyroid and pituitary glands fixed in 10 per cent formalin solution Paraffin embedded sections were stained with haematoxylin-eosin and van Gieson's stain Sections of liver and spleen were also stained for haemosiderin with Turnbull's blue method. Frozen sections of liver lungs heart, spleen, lymph nodes kidneys and adrenals were stained with Scharlach Rot and Sudan Black B and also analysed with the polarizing microscope In addition, sections of the thyroid gland were stained with periodic acid Shiff (PAS) and sections of the pituitary gland with trichrome periodic acid Shiff according to *Pearse* (10) Serum cholesterol levels were determined according to a modified Liebermann Burchard reaction.

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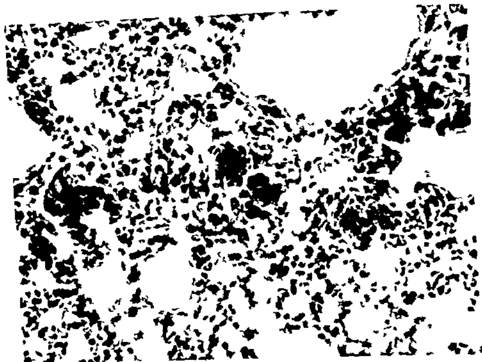
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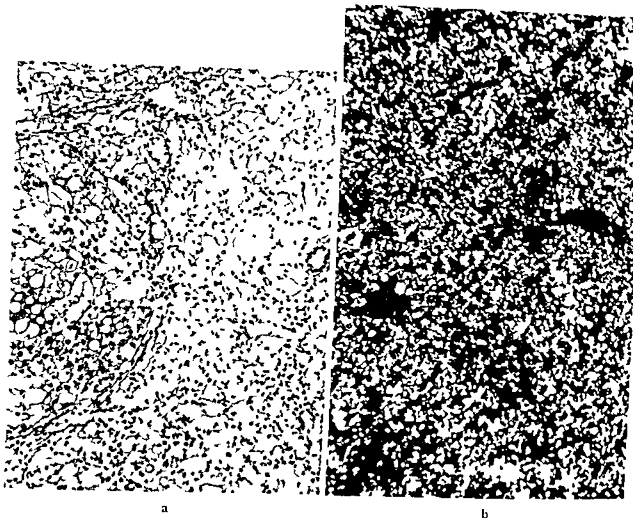
F g 3 Lung from gerb l fed cholesterol cholic acid d et for 4 months Clusters of fat posi t e al eolar macrophages Frozen section Scharlach Rot × 320

1) Large accumulations of small whitish gallstones were found in 5 of the 7 survivors Sim lar gallstones had been present in most of the animals which had died during the last month of the experiment Chemical ana lysis of these stones showed them to consist of cholesterol No gallstones were present in the controls nor have such stones ever been observed in dead animals from our stock colony The spleen was markedly enlarged and ascites was present in all the surviving test group animals

Histological examination of the liver re vealed an extreme fatty degeneration with massive accumulation of intracellular lipid droplets and birefractive cholesterol crystals in the parenchymal and Kupffer cells The l ver cells showed polymorphism and many b nuclear cells were present A slight fibrosis and nodular parenchymal regeneration were observed (Figs 2 a–b)

The gallbladder had a thin wall with slight f brosis and atrophy of the mucous mem brane In some animals small mucosal ulcer ations and scattered infiltrates of inflamma tory cells were found

A marked accumulation of stainable lipids and birefractive cholesterol crystals were also found in the reticulo endothelial cells of lungs spleen and lymph nodes In the lungs, clusters of fat positive alveolar macrophages were found freely in the alveolar lumen or attached to the alveolar wall (Fig 3) In the spleen the parenchymatous tissue was in large areas virtually replaced by lipid con taining histiocytes and accumulations of needle like crystals and granules (Figs 4 a–b) There was also an increase in Turn bull positive substance in the spleen of the animals given the supplemented diet In some cholesterol cholic acid treated animals the kidneys showed slight fatty changes in the loop of Henle and in the proximal con voluted tubules As for the endocrinal glands the dietary supplement seemed to accentuate the hypoplasia of the thyroid gland found in the normal controls No difference was found in the anterior pituitary gland while there



Figs 2 a b Liver from gerbil fed cholesterol cholic acid diet for 4 months
a) marked steatosis with cell polymorphism and slight fibrosis v Gieson × 120
b) same specimen as in 2 a Massive accumulation of birefractive cholesterol crystals Frozen section Polarized light × 50

group They were individually caged and had free access to food and water They were fed during one month with chow supplemented with 1 per cent cholesterol and 0 5 per cent cholic acid After one month 20 animals were chosen in a strictly random fashion sacrificed and investigated For the remaining animals of the experimental group the diet was switched back to unsupplemented chow One (1) 2 and 3 months later 20 gerbils chosen at random were sacrificed and examined as described above In the group of animals sacrificed after 3 months on chow no analysis of blood and bile was performed as the studied variables had already returned to normal

The histological examinations were performed after the whole experiment had been completed the assessor being unaware of the group to which the animal belonged

RESULTS

Experiment 1 (4 Months Exposure)

After 4 months there were 7 survivors in the test group as compared with 6 among the controls The mean weight of the surviving test group animals had risen to 75 1 g as compared with 72 8 g of the controls The mean liver weight of the cholesterol cholic acid fed gerbils was 11 1 g that of the controls 3 1 g When subjected to the Mann Whitney U test (14) this latter difference was highly significant The liver of the surviving test group animals had the pale yellowish colour typical of extreme steatosis and the gallbladder was extremely dilated (Fig

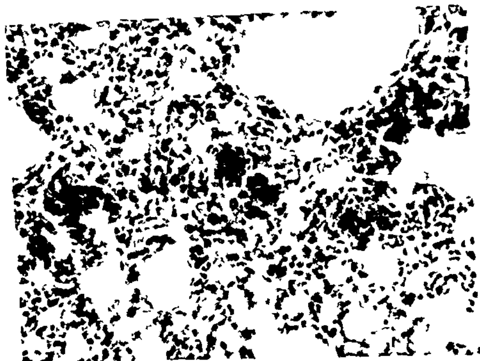


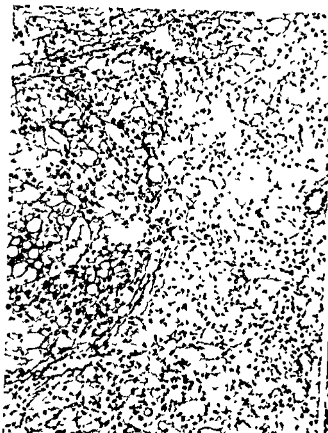
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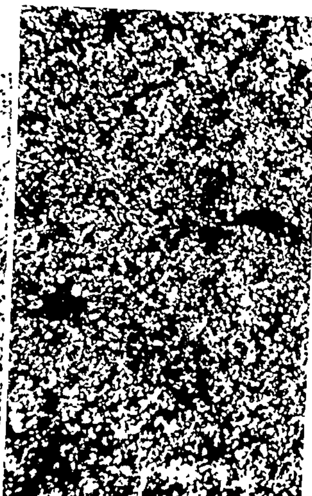
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a.



b

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a) marked steatosis with cell polymorphism and slight fibrosis v. Gieson × 120
b) same specimen as in 2 a Massive accumulation of birefractive cholesterol crystals Frozen section Polarized light × 50

group They were individually caged and had free access to food and water They were fed during one month with chow supplemented with 1 per cent cholesterol and 0 5 per cent cholic acid After one month 20 animals were chosen in a strictly random fashion, sacrificed and investigated For the remaining animals of the experimental group the diet was switched back to unsupplemented chow One (1), 2 and 3 months later, 20 gerbils chosen at random were sacrificed and examined as described above In the group of animals sacrificed after 3 months on chow no analysis of blood and bile was performed as the studied variables had already returned to normal

The histological examinations were performed after the whole experiment had been completed, the assessor being unaware of the group to which the animal belonged

RESULTS

Experiment 1 (4 Months Exposure)

After 4 months there were 7 survivors in the test group as compared with 6 among the controls The mean weight of the surviving test group animals had risen to 75 1 g as compared with 72 8 g of the controls The mean liver weight of the cholesterol cholic acid fed gerbils was 11 1 g, that of the controls 3 1 g When subjected to the Mann-Whitney U test (14) this latter difference was highly significant The liver of the surviving test group animals had the pale yellowish colour typical of extreme steatosis and the gallbladder was extremely dilated (Fig

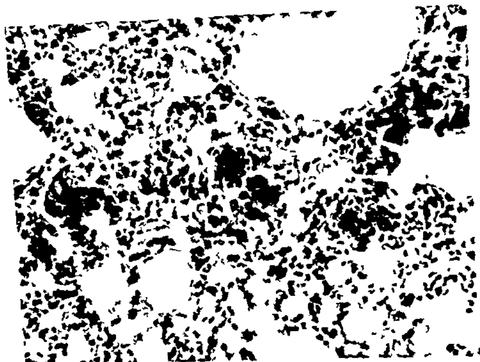


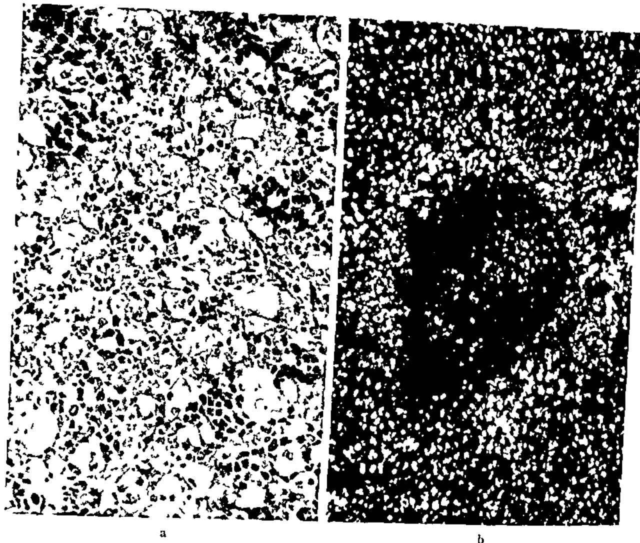
Fig 3 Lung from gerbil fed cholesterol cholic acid diet for 4 months Clusters of fat positive alveolar macrophages Frozen section Scharlach Rot × 320

1) Large accumulations of small whitish gallstones were found in 5 of the 7 survivors Similar gallstones had been present in most of the animals which had died during the last month of the experiment Chemical analysis of these stones showed them to consist of cholesterol No gallstones were present in the controls nor have such stones ever been observed in dead animals from our stock colony The spleen was markedly enlarged and ascites was present in all the surviving test group animals

Histological examination of the liver revealed an extreme fatty degeneration with massive accumulation of intracellular lipid droplets and birefractive cholesterol crystals in the parenchymal and Kupffer cells The liver cells showed polymorphism and many binuclear cells were present A slight fibrosis and nodular parenchymal regeneration were observed (Figs 2 a–b)

The gallbladder had a thin wall with slight fibrosis and atrophy of the mucous membrane In some animals small mucosal ulcerations and scattered infiltrates of inflammatory cells were found

A marked accumulation of stainable lipids and birefractive cholesterol crystals were also found in the reticulo endothelial cells of lungs spleen and lymph nodes In the lungs, clusters of fat positive alveolar macrophages were found freely in the alveolar lumen or attached to the alveolar wall (Fig 3) In the spleen the parenchymatous tissue was in large areas virtually replaced by lipid-containing histiocytes and accumulations of needle like crystals and granules (Figs 4 a b) There was also an increase in Turnbull positive substance in the spleen of the animals given the supplemented diet In some cholesterol cholic acid treated animals the kidneys showed slight fatty changes in the loop of Henle and in the proximal convoluted tubules As for the endocrinal glands the dietary supplement seemed to accentuate the hypoplasia of the thyroid gland found in the normal controls No difference was found in the anterior pituitary gland while there



Figs 4 a–b Spleen from gerbil fed cholesterol cholic acid diet for 4 months
a) replacement of tissue by lipid containing macrophages Htx eosin × 290
b) accumulation of birefractive cholesterol crystals In centre, a Malpighian corpuscle with only scattered crystals Frozen section Polarized light × 50

was a marked increase in stainable lipids and cholesterol crystals in all layers of the adrenal cortex of the animals given the supplemented diet (Figs 5 a–b) No atherosclerotic changes were ever found during the experimental period

Comparison of the serum cholesterol levels revealed extreme differences with a mean of 867 mg per cent in the test group as compared with one of 104 mg per cent in the controls

Experiment 2 (Regression after 1 Month Exposure)

Also the gerbils sacrificed after one month on the cholesterol cholic acid supplemented diet showed a conspicuous enlargement of the liver which as a rule had a pale yellowish colour The histological picture was dominated by the accumulation of intracellular lipid droplets and of birefractive cholesterol crystals in the parenchymal and Kupffer cells No gallstones were present and the gallbladders were only moderately distended In the gerbils which were sacrificed one two and four months after the diet had been switched back to normal similar changes although of a lesser degree were observed in the liver The fatty changes persisted chiefly in the central and midzones of the lobules (Fig 6) The other histological changes observed after 1 month on the supplemented



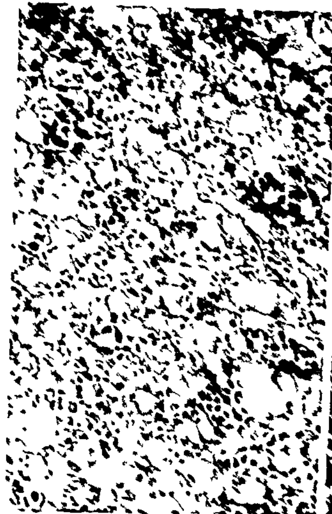
Figs 5 a b Adrenal cortex from gerbil Htx-eosin × 100
a) (chow diet) large lipid laden cortical cells, particularly in zona fasciculata
b) (cholesterol cholic acid diet) increased occurrence of lipids in all cortical zones

diet were of a similar nature but less marked than those observed after 4 months on that diet They disappeared when the diet was switched back to normal chow In the thyroid gland a moderate fibrosis was left behind

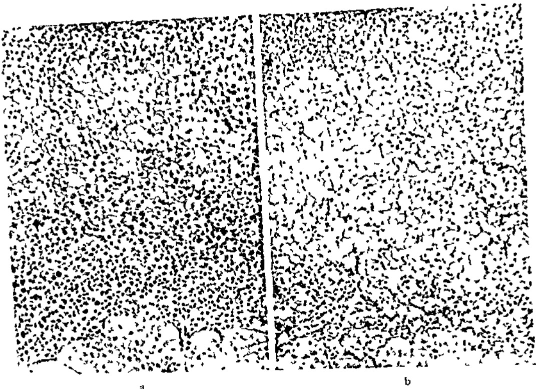
After one month on the supplemented diet the mean liver weight had risen from 2.5 g to 3 9 g, a difference which was significant with the Mann Whitney U test (14) This difference decreased when the diet was switched back to normal However, even four months later the liver weight still differed significantly from that found in the controls

After one month on the supplemented diet the serum cholesterol value had risen to nearly the same very high levels as those reached in Exp 1 after four months (see Fig 7) Switching the diet back to chow resulted in a rapid decrease of these levels and after 2 months the mean level was about the same as pre experimentally

The cholesterol concentration of the bile rose to roughly twice the original value after one month on the supplemented diet (see Fig 8) Discontinuance of this diet was followed by a gradual decrease, the pre-experimental value being reached after 2 months As for the bile acid composition, after the supplemented diet had been given during one month we found a significant rise of the cholic acid and deoxycholic acid concentrations whereas the chenodeoxycholic acid concentration showed a significant decrease Already one month after the diet had been switched back to chow, these changes had disappeared (see Fig 9) Finally, no significant differences were observed for phospholipids the mean value observed in the gerbils sacrificed after one month on the supplemented diet being somewhat higher than that in the other animals







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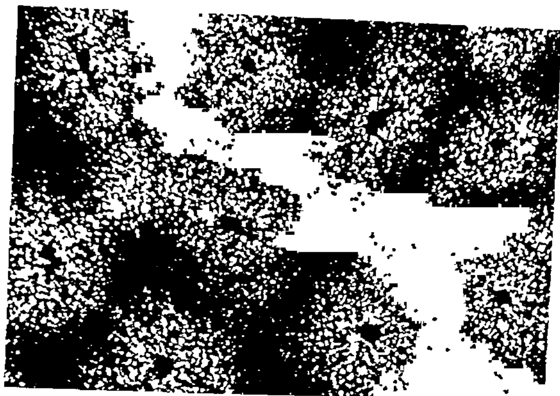


Fig 6 Liver from gerbil fed cholesterol cholic acid diet for one month and then chow diet for four months Deposition of birefractive cholesterol crystals and granules in the central and midzones of the lobuli Frozen section Polarized light × 35

DISCUSSION

The mongolian gerbil is a relatively new but potentially very useful laboratory animal After the first pairs of the species had been caught in Eastern Mongolia in the midthirties they have been bred in Japan where deliberate efforts have been made to develop them as an experimental animal As such they have proved to have rather unique qualities Most important they require very little labour and their susceptibility to infection is reported to be low (12)

Attention has been drawn to the gerbil s reaction to the administration of cholesterol in the diet (7) On such a diet they develop high serum and hepatic cholesterol levels (8) *i e* a response similar to that found in for instance rabbits and chickens but in contrast to these latter animals they develop no gross atherosclerotic changes Data have been brought forward suggesting that the formation of steroid hormones may be a more important pathway in the gerbil s cholesterol metabolism (13) Furthermore its gastro-intestinal absorption of cholesterol seems to be enhanced as compared with the rat (8) As these data seem to indicate that cholesterol metabolism in the gerbils may differ from that in other animals we thought it of interest to study its reaction to different gallstone provoking diets We found *i a* that a fatfree diet rich in glucose which induces gall stones in hamsters did not have any effect in gerbils On the other hand as shown by the results reported here gallstones can be induced in gerbils by feeding a cholesterol cholic acid containing atherogenic diet which induces gallstones in mice (15) Analysis of the bile of gerbils fed the cholesterol cholic acid diet during one month showed the mean cholesterol concentration to be about twice the pre-experimental value The total bile acid concentration rose only slightly with this diet and so did the phospholipid concentration As a result the total bile acid/cholesterol ratio and the phospholipid/cholesterol ratio fell considerably This is in agreement with findings in mice when gall

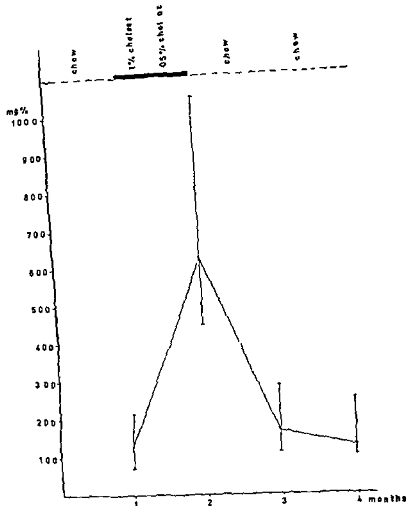


Fig 7 Serum cholesterol level (mean and range) under different dietary conditions

stones are formed with the same gallstone inducing diet (4) Similar findings in hamsters when these animals were given a gallstone inducing diet of a totally different composition have been reported (11) The morphological changes in gerbils fed the cholesterol cholic acid containing diet were dominated by steatosis of the liver and accumulation of lipids in the reticuloendothelial system The changes were even more accentuated than those in hamsters and mice fed the same diet and previously described (2) The accumulation of macrophages in the gerbils spleen suggests that these cells participate actively in the removal of lipid and cholesterol The reaction of the gerbils gallbladder to the cholesterol cholic acid supplemented diet is much like that of the mouse In both species there is a sometimes very marked distention of the gallbladder with signs of inflammation in some animals Another point of similarity with the mouse is the reaction of the thyroid and adrenal glands In both species we found with the atherogenic diet a marked accumulation of stainable lipids in the adrenal cortex and hypoplasia in the thyroid gland In

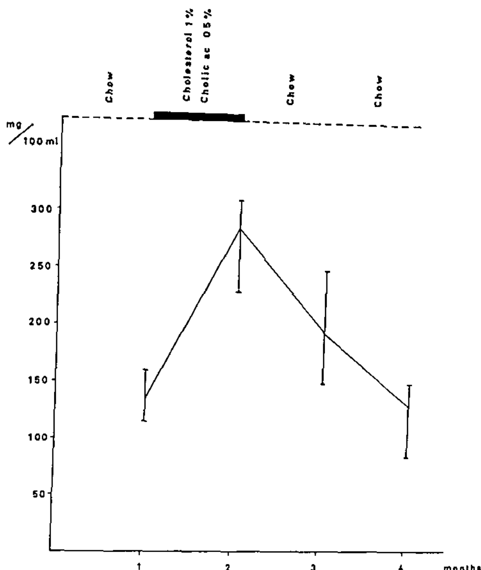


Fig 8 Cholesterol concentration in bile (mean and range) under different dietary conditions

the gerbil considerable hypoplasia was also present in control animals but the hypoplastic changes seemed to be more accentuated in the animals fed the supplemented diet In this latter respect both species react as the rabbit and guinea pig and unlike the hamster and the rat on an atherogenic diet in which cases hyperplasia is found to develop It has been pointed out that atherosclerotic changes occur only in species in which thyroid hypoplasia develops on an atherogenic diet (3) The gerbil does however, not follow this pattern It reacts with thyroid hypoplasia as found here, but is known not to develop atherosclerotic changes This makes a connexion between these two phenomena less likely

The serum cholesterol values attained extremely high levels in the gerbil already after one month on the cholesterol cholic acid supplemented diet The increase observed in the gerbil is much more marked than in mice and hamsters fed with the same diet

The bile acid pattern of the gerbil was found to be much similar to that of the mouse (6) In the gerbil bile acid consists mainly of cholic acid with only small amounts of chenodeoxycholic- and deoxycholic acid Just as in the mouse and the hamster feeding the cholesterol cholic acid diet resulted in a

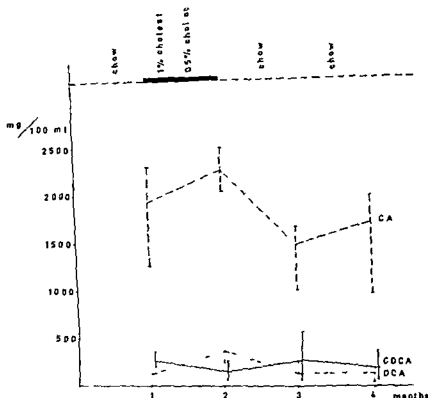


Fig 9 Concentrations of principal bile acids (means and ranges) under different d etary conditions

decrease of the chenodeoxycholic acid con centration (2) The probable reason is a diminished synthesis of bile acids due to the continuous supply of cholic acid via the diet The deoxycholic acid concentration showed a small rise with the diet In the hamster we found a *striking rise while this concentration* was unchanged in the mouse These differ ences may reflect specie differences in the ability to rehydroxylate deoxycholic acid Just as in the mouse and in contrast to the ham ster already one month after discontinuance of the diet the bile acid pattern had returned to normal

In one respect, however there is a striking difference between the gerbil and the mouse When the diet was discontinued, liver histology returned rapidly to normal in the latter species (1) whereas it diminished only gradually in the gerbil and was not normal even after 4 months Although the cholesterol concentration in serum and bile had returned to normal already after 2 months, the histological examination of the liver showed that the gerbil has great difficulty in freeing itself from the excess accumulation of cholesterol *In this respect the gerbil is com* parable to the hamster In this latter species we found in a similar experiment liver histology to be largely unchanged two months after discontinuance of the cholesterol cholic acid containing diet (2)

Apart from this marked difference between the gerbil and the mouse in their ability to rid themselves of the accumulation of excess cholesterol, their reaction to the cholesterol cholic acid containing diet was much the same It seems likely therefore that gallstone formation observed in the gerbil fed this diet, occurs in the same way as in the

mouse However, because of its poor tolerance to the diet when it is given during a long period of time, the gerbil seems a less suitable model for study

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Acta path microbiol scand Section A 79, 487 490 1971

ABORTION IN MICE INDUCED BY ELLAGIC ACID

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Ellagic acid was injected intravenously into mice at day 8, 12, or 16 of pregnancy In all animals given this treatment there was an increased frequency of abortion Platelet fibrin masses were found in the lungs, in the great majority of the mice given ellagic acid at day 16 of gestation The study seems to show that there is a tendency to thrombosis in pregnancy of the mouse

In a previous study, intravenous injection of ellagic acid, an activator of factor XII (Hageman factor) (6), was found to induce *formation of platelet fibrin masses in placentas* of 16 days pregnant mice (4) This was followed by necrosis of most placentas However, the frequency of abortion induced by ellagic acid was not explored The purpose of the present study was to determine the effect of intravenous injection of ellagic acid on the outcome of pregnancy in mice Mice were therefore given ellagic acid at different stages of pregnancy and the number of abortions was determined Further, as an indicator of the tendency of thrombosis produced by the ellagic acid the presence of platelet fibrin masses in the lungs was investigated

MATERIALS

Animals Altogether 80 virgin female non inbred albino mice were used They were 55 57 days old and weighed 24 33 g (mean 28 8 g) The animals were kept under artificial light in plastic cages the floor of which was covered by saw dust They were fed a standard diet (SIFF Norwegian standard stock diet no 1, mice and rats*) and tap water *ad libitum* Animals taken out for mating were kept single, and the males were put in with the females in the morning After 24 hours the males were removed, that day was designated day one of pregnancy or day one after mating if the animal showed increasing weight At day 8 12 and 16 the mean body weight had increased 4 6 g, 6 6 g and 14 2 g, respectively *Ellagic acid* Supplied by K and K Lab, Inc, New York, U S A The ellagic acid was dissolved in buffered saline to a concentration of 4×10^{-4} M as described by *Nordoy & Chandler* (5) *Buffered saline* A 0 9 per cent solution of NaCl in distilled water was buffered with 10 per cent tris (hydroxymethyl) aminomethane HCl, 0 15 M to a final pH 7 2, and used as diluent

METHODS

Anaesthesia The animals were anaesthetized by intraperitoneal injection of *Nembutal Veterinary* (Abbott Lab Ltd Queenborough Kent, England) 5 mg per 100 g body weight

Experimental procedure At day 8, 12 or 16 of pregnancy (10 mice in each group) laparotomy

Received 8 ii 71

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*) Grained barley 35 per cent, grained oat 15 per cent, grained wheat 12 per cent, soy meal 12 per cent, herring meal 10 per cent, grass meal 5 per cent dried skimmed milk 10 per cent, salt and mineral and vitamin mixtures 1 per cent

mouse However, because of its poor tolerance to the diet when it is given during a long period of time, the gerbil seems a less suitable model for study

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TABLE 3 *The Number of Mice in Which Masses Composed of Platelets and Fibrin Were Found in the Pulmonary Arteries and Veins*

Treatment	Frequency of platelet fibrin masses
Ellagic acid (8th day)	0/10
Ellagic acid (12th day)	1/10
Ellagic acid (16th day)	9/20
Saline	1/40

The great majority of them was found in mice treated with ellagic acid on the 16th gestational day

DISCUSSION

In the present study ellagic acid was given intravenously to mice at day 8, 12, or 16 of pregnancy In all animals given this treatment there was an increased frequency of abortion but only in mice injected on the 16th gestational day was the prevalence of abortion significantly higher compared with the controls The difference in frequency of abortion between the groups may be caused by a different reaction upon the laparotomy, this difference is also found between the control groups

Abortion is probably caused by necrosis of the placenta and the necrosis is again produced by thrombi The following facts support this theory Ellagic acid is found to activate factor XII (Hageman factor) (6) of the intrinsic coagulation system Injection of ellagic acid into non pregnant animals of species other than mouse does not cause thrombosis but causes shortening of bleeding time (2 3 5) and whole blood clotting time (1 2) However ellagic acid injected intravenously into pregnant mice at day 16 of gestation is found to produce considerable amounts of platelet fibrin masses in the maternal blood channels of the placenta and 24 hours after the injection most of the placentas showed necrotic changes (4) As discussed previously (4), the factor most important for the tendency to thrombosis in the placenta may be the particular flow pattern in the organ, and this factor may be the additional stimulus necessary for ellagic acid to induce thrombosis Furthermore, when ellagic acid was injected on day 16 of gestation platelet fibrin masses occurred in the lungs That all platelet fibrin masses are emboli from the placenta seems unlikely since they were found in the veins as well as in the arteries Another possibility is that the platelet fibrin masses in the lungs are induced by a liberation of a clot promoting substance from the placenta (7, 8) This theory is also unlikely since platelet fibrin masses were found in the lungs already 3 minutes after the intravenous injection of ellagic acid into 16 days pregnant mice, and at this time the placentas were morphologically unaltered (4)

The platelet fibrin masses were much less frequent in the lungs of animals injected on the 8th or 12th day than when ellagic acid was given on day 16 of pregnancy This may in part be caused by a different reaction upon the laparotomy Furthermore, if the effect of ellagic acid was the same in all animals one may also consider that the frequency of platelet fibrin masses in the lungs would be dependent upon the duration of the period between the injection of ellagic acid and the sacrifice of the animals

Thus, the injection of ellagic acid in mice unveil a general tendency to thrombosis in pregnancy not present in the non pregnant mouse (4) However, organ differences seem to exist, as the tendency to thrombosis is more extensive in the placentas than in the lungs

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TABLE 1 *Effect of Intravenous Injection of Ellagic Acid on Pregnancy in Mice*

Day of treatment	Treatment	No of pregnant mice	Mean no of foetuses	Mean no of living offsprings 22 day	Mean per cent loss ±S E
8th	E a + lap	10	9 9	4 9	52 7±10 6
	Saline + lap	10	10 3	7 4	28 4± 9 9
12th	E a + lap	10	8 1	5 1	38 7±12 3
	Saline + lap	10	7 8	5 6	26 7±10 0
16th*	E a + lap	10	10 0	2 8	72 4±11 2
	Saline + lap	10	8 2	5 1	38 4±11 8

E a , ellagic acid injection, Lap , anaesthesia + laparotomy,
S E , standard error of the mean
* *Difference between mean loss* $p=0\ 05$

was performed and the number of foetuses was counted Thereafter the incision was closed, and ellagic acid was given into the tail vein in a dosage of 1 ml (4×10^{-4} M) per 100 g body weight over a period of 30 seconds

In control animals, a comparable volume of buffered saline was substituted for the solution of ellagic acid

After the operation the animals were weighed daily At day 22 after mating (normal pregnancy duration is 18 to 21 days) the living offsprings were numbered, the mother sacrificed, and the lungs removed for histological examination The uterus was inspected, in no cases products of foetal origin were found In the period from the day of injection to day 22 after mating, abortion products were often seen in the cages These observations were not registered because the mother often eats up the abortion products If the mother lost a large number of foetuses, this could be recorded by an explicit drop in weight

To eliminate the abortion effect of the operation trauma, another experiment was performed The diagnosis of pregnancy is certain at day 16 because of a considerable gain in weight but without laparotomy the number of foetuses is unknown Therefore, 16 days pregnant mice were given ellagic acid or buffered saline under anaesthesia but without preceding laparotomy (10 animals in each group) At day 22 after mating, instead of numbering the offsprings, mothers with one or more living offsprings and mothers with all foetuses lost were recorded

No unintended deaths occurred among the animals

Staining and examination of histological sections The sections from the lungs were stained with haematoxylin erythrosin saffron They were examined without knowledge of the treatment given to the animal in question

TABLE 2 *Mice Treated with Intravenous Injection of Ellagic Acid on the 16th Gestational Day*

Treatment	No of pregnant mice	No of mice with all foetuses lost
Ellagic acid + anaesthesia only	10	4
Saline + anaesthesia only	10	0

Fisher exact probability test $p=0\ 04$

RESULTS

Table 1 shows that in animals on which laparotomy and injection of ellagic acid had been performed on the 8th or 12th gestational day, there was a slightly higher loss of foetuses in the ellagic acid treated group than in the buffer-treated group These differences were not significant In animals treated on the 16th day, however, the mean foetal loss in the ellagic acid treated group was significantly higher

Table 2 shows the number of pregnant mice in which laparotomy was not performed and in which all foetuses were lost after injection of ellagic acid or buffered saline on the 16th gestational day The difference is between the two groups is statistically significant

Table 3 shows the number of the animals in which platelet-fibrin masses were found in the pulmonary veins and arteries The platelet-fibrin masses were in general scarce

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METASTASES TO THE MALE BREAST

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In an autopsy series of 16 294 subjects 7 165 carcinomas were demonstrated. Among 2 195 metastasizing carcinomas in men 22 (1 per cent) were found to have metastases to the breast. The autopsy series contained 2 men with breast lesions diagnosed as primary The primary tumour most often metastasizing to the breast was oestrogen treated prostatic carcinoma (20 of the 22 cases) Mammary tumours in patients with oestrogen treated prostatic carcinoma are probably metastases rather than independent primary tumours because oestrogen therapy had usually been given for a short time and in relatively small doses oestrogen therapy by induction of gynaecomastia may render the breast a preferential site of metastasis the lesions of the breast are often multiple and bilateral the lesions of *the breast generally occur in patients with generalized* spread to several lymph node stations and other organs and careful histological examination often shows the same histological picture as the prostatic carcinoma

In a patient with metastasizing primary tumour elsewhere it may be difficult to decide whether a tumour of the breast is primary or secondary This applies in particular to patients with prostatic cancer treated with oestrogens and in which the possibility of a hormone induced primary tumour of the breast cannot be excluded Thorough investigations (*Benson* 1957 *Pribe* & *Ockuly* 1963) have shown that in such patients lesions of the breast are as a rule metastases Most publications on metastases to the male breast are however based on descriptions of single cases or of compilations from the literature The large monographs by *Walther* (1948) and *Willis* (1962) for example, give no personal cases The largest personal series on record is reported by *Charache* (1953) and consists of 4 cases It was therefore considered desirable to report on the frequency of metastases to the male breast in a large autopsy series examined according to uniform principles Since it proved that prostatic carcinoma was the preponderant type of primary tumour in such cases, this tumour will receive most attention in the following

MATERIAL AND METHODS

Malmo has a well defined population about 250 000 and constitutes a separate area of the health service There is one general hospital, one for chronic *diseases and one for mental diseases* all served by one institute of pathology

The basic material consisted of all subjects of all those who had died in hospital) All autopsies were performed according to uniform principles throughout the period in question The methods have been described in some detail earlier (*Berge* 1967) The breast was not *examined* histologically

Received 16 ii 71

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TABLE 1 *Total Number, Number and Frequency of Metastasizing Tumours and Number of Organs with Metastases*

te of rimary tumour	Number of tumours			Metastasizing tumours						Number of organs to which metastasizing tumours had spread: Lymph node groups	Other organs	Other organs excl serous cavities
	f	m	tot	f	%	m	%	tot	%	tot	tot	tot
ye	6	10	16	6	100	9	90.0	15	93.8	2.3	8.7	7.7
Inknown	39	26	65	39	100	26	100	65	100	2.5	3.6	2.9
ongue	3	7	10	2	66.7	2	28.6	4	40.0	2.8	1.3	1.0
weat gland	1	1	2	1	100	0		1	50.0	4.0	6.0	3.0
Maxillary sinus	4	8	12	1	25.0	4	50.0	5	41.7	1.6	1.6	1.2
Nasal cavity	1	0	1	1	100	0		1	100	0	8.0	7.0
pipharynx	1	7	8	1	100	7	100	8	100	1.9	1.6	1.5
arynx	0	30	30	–		13	43.3	13	43.3	1.8	2.4	1.9
Trachea	1	0	1	1	100	–		1	100	1.0	0	0
Lung	178	569	747	139	78.1	505	88.8	644	86.2	2.5	3.1	2.7
ip	3	11	14	2	66.7	3	27.3	5	35.7	0.8	0.8	0.8
Oral cavity	6	12	18	3	50.0	8	66.7	11	61.1	2.1	2.1	1.6
Salivary gland	7	3	10	4	57.1	1	33.3	5	50.0	2.6	4.0	3.8
Tonsil	2	2	4	1	50.0	2	100	3	75.0	2.3	0.3	0.3
Hypopharynx	4	10	14	2	50.0	5	50.0	7	50.0	1.9	1.4	1.1
Oesophagus	34	76	110	25	73.5	58	76.2	83	75.5	1.9	1.8	1.5
Cardia	1	2	3	1	100	2	100	3	100	1.0	3.0	2.0
Stomach	278	373	651	231	83.1	302	81.0	533	81.9	2.7	2.1	1.4
Small intestine	57	102	159	21	36.8	27	26.5	48	30.2	1.2	0.8	0.7
Appendix	8	11	19	2	25.0	5	45.5	7	36.8	1.3	1.9	0.7
Colon	361	344	705	223	61.8	185	53.8	408	57.9	1.7	2.3	1.8
Rectum	125	189	314	79	63.2	111	58.7	190	60.5	1.7	2.4	2.0
Anus	10	3	13	7	70.0	2	66.7	9	69.2	2.2	1.9	1.6
Liver	75	196	271	47	62.7	122	62.3	169	62.4	1.9	2.0	1.6
Biliary system	169	79	248	147	87.0	61	77.2	208	83.9	2.2	1.8	1.3
Pancreas	118	179	297	108	91.5	169	94.4	277	93.3	2.0	2.5	1.8
Thyroid	42	25	67	23	54.8	19	76.0	42	62.7	1.9	2.9	2.6
Adrenal	2	3	5	2	100	3	100	5	100	1.4	1.8	1.8
Kidney	138	218	356	63	45.7	100	45.9	163	45.8	1.3	3.1	2.6
Renal pelvis + ureter	18	16	34	17	94.5	12	75.0	29	85.3	1.6	2.8	2.6
Urinary bladder + urethra	56	158	214	30	53.6	83	52.5	113	52.8	2.3	2.3	1.9
Testis		18	18			15	83.3	15	83.3	2.3	4.3	3.5
Prostate		1332	1332	–		299	22.4	299	22.4	2.8	2.3	2.0
Penis		9	9	–		2	22.2	2	22.2	1.5	1.0	0.5
Ovary	253		253	217	85.8	–		217	85.8	3.4	2.7	1.5
Tube	7		7	6	85.7	–		6	85.7	2.2	1.8	0.7
Uterine body	111		111	49	44.1			49	44.1	1.8	2.4	1.7
Uterine cervix	181		181	109	60.2			109	60.2	2.5	2.4	1.9
Uterus unspec	13		13	11	84.6			11	84.6	2.9	3.4	2.6
Vagina	7	–	7	4	57.1	–		4	57.1	2.3	2.0	1.5
Vulva	23	–	23	17	73.9	–		17	73.9	2.4	3.9	3.0
Breast	693	2	695	539	77.8	2	100	541	77.8	4.3	3.8	3.1
Skin	44	54	98	28	63.6	31	57.4	59	60.2	3.2	8.1	7.2
Total	3080	4085	7165	2209	71.7	2195	53.7	4404	61.5	2.5	2.7	2.2

in all males with gynaecomastia and histo chemical determinations of the acid phosphatases were not performed

The data were treated at the computer in Lund

RESULTS

Among the 16,294 autopsies performed during the 12-year period altogether 8,214 malignant tumours were found in 7,246 subjects (44 5 per cent of all autopsies) Among the tumours, 7,165 were carcinomas, while the remaining 1,049 lesions consisted of leu cosis, malignant lymphoma, tumours of the central nervous system and different types of sarcoma All cases with metastases to the male breasts had malignant epithelial tumours and the distribution of the primary carcinomas among the various organs involved is given in Table 1

Malignant growths in the male breast were demonstrated in 22 cases or in 1 per cent of all metastasizing carcinomas in males In 20 cases the primary tumour was situated in the prostate (6 7 per cent of the metastasizing prostatic carcinomas), while the remaining 2 were cases of adenocarcinoma of the lung in a 53-year old man and choriocarcinoma of the testis in a 23 year old man During the same 12 year-period 2 cases of primary cancer of the breast were diagnosed in males

Out of the 1 332 cases of carcinoma of the *prostate*, 299 (22 4 per cent) had metastasized The remaining cases were mostly cases of latent cancer which had been demonstra ted at routine histological examination The number and frequency of secondary growths set up in various organs by prostatic carcinomas are grouped according to histological type *in Table 2*

The tumours of the breast were bilateral in 16 cases (prostatic cancer in 15 cases, choriocarcinoma of the testis in 1) The 6 unilateral cases were all confined to the left side All 20 cases of prostatic cancer had been treated with oestrogens and they had all developed gynaecomastia (gynaecomastia was also seen in the case of choriocarcinoma!) On the average, treatment with oestrogens had been given for 25 months (range 8 months to 7 years)

Most of these cases showed generalized spread of the tumour Among 299 cases of metastasizing prostatic carcinoma the disease had on the average spread to 2 8 lymph node stations and 2 3 other organs Among cases with tumour of the breast the mean values were 4 7 lymph node stations and 5 2 other organs All 20 cases had metastases to lymph nodes and 19 of them also had demonstrable skeletal metastases (Among all metastasizing prostatic carcinomas, 87 3 per cent had lymph node—and 78 6 per cent skeletal involvement)

Tumours of the breast were diagnosed at gross and microscopical examination in 13 cases (including 11 with prostatic cancer), while the remaining 9 cases were discovered only at microscopical examination unfortu nately not performed in all cases of gynaeco mastia (In a random selection of 25 cases of metastasizing prostatic cancer treated with oestrogens and without demonstrable tumour of the breast, 22 had gynaecomastia In 2 cases the autopsy protocols contained no notes about gynaecomastia and in 1 case the examiner had excluded gynaecomastia Out of these 25 cases, only 7 had been examined histologically)

The histological distribution of the cases with secondary growth in the breast *was the* same as that of those without The histological picture of the prostatic tumours *in*variably agreed with that of the breast lesions

DISCUSSION

Gross examination often decides whether a lesion is a primary tumour or a metastasis This is less often so in patients with prostatic cancer and tumour of *the breast* because of the possibility of a primary tumour induced in the breast by treatment with oestrogens *This problem* has been studied among others by *Foote & Stewart* (1945) who claim that *no certain* case of oestrogen induced *mam*mary cancer in man has been described The

a tumour of the breast in the first month of treatment with diethylstilbestrol, 50 mg a day Many investigators (for ref see *Pribe & Ockuly* 1963) feel that oestrogen has no carcinogenic effect on the male breast

Patients with cirrhosis of the liver often develop gynaecomastia but very seldom breast cancer It has, however, been claimed that gynaecomastia caused by oestrogen therapy can change the mammary tissue to make it a preferential site of metastasis This would also explain why the tumours of the breast in these cases are so often bilateral Another possible contributory factor of the high frequency of metastases is the prolongation of survival by oestrogen therapy

If oestrogen changes the mammary tissue favouring the development of metastases, it should also provide a possibility of demonstrating this, for example in patients with gynaecomastia because of liver cirrhosis This was as mentioned, not the case in the Malmö series perhaps because patients with liver cirrhosis apart from primary liver cancer, have a lower cancer frequency than others and often die earlier than patients with tumour without cirrhosis The number of cases with generalized spread is therefore small (for ref see *Berge & Saldeen* 1968)

That tumours of the breast are usually seen in patients with generalized spread has been emphasized e g by *Campbell & Cummins* (1951) and is also apparent from this series in which the patients with tumour of the breast had metastases to twice the average of organs in all patients with tumours of the prostate (The 2 patients with tumours of the lung and testis, respectively, had besides lymph node metastases, metastases in a further 6 organs or organ systems) Also this suggests that the mammary tumours are secondaries

No specimens were examined histo-chemically for acid phosphatases In those cases where such determinations are reported (for ref see *Lome & Austen* 1970) they were said to support the metastatic nature of the tumours The value of histo-chemical examination may, however, be questioned Lysosomal enzymes such as acid phosphatases may occur also in tumours other than those originating in the prostate *Glück* (1952) who used Gomori's technique, examined mammary cancers from 33 women With only few exceptions he found acid phosphatases in the tumour cells Quantitative determinations, as used by *Campbell et al* (1961) may facilitate differentiations

The 22 cases described constitute only 1 *per cent* of all metastasizing carcinoma in men A search of the literature failed to reveal any suitable series for comparison *Abrams et al* (1951), who examined 1,000 consecutive cases of cancer, reported a frequency of 5 per cent for metastases to the breast Most of their patients, however, were women

While 13 of the present cases were recognized at gross examination, the metastases in 9 were found at the histological examination because of gynaecomastia Among the 22 cases in the control series for gynaecomastia, only 7 were examined microscopically The frequency found in the present series is therefore surely a minimum figure

Recognition of cases of metastases to the breast would require routine histological examination of all cases of cancer Owing to the close association with gynaecomastia, it should in practice be sufficient to confine the examination to such cases Whether histo-chemical examination for acid phosphatases would facilitate distinction between primary and secondary tumours in the male breast is still an open question

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TABLE 2 *Number and Frequency of Metastases from Primary Tumours of the Prostate According Histological Type*

Site of metastases	Histological type					
	Adeno-carcinoma		Undiff carcinoma		Total	
	No	%	No	%	No	%
Brain	6	2 7	1	1 3	7	2 3
Meninges	12	5 4	6	7 8	18	6 0
Trachea			1	1 3	1	0 3
Lung	100	45 0	39	50 6	139	46 5
Pleura	40	18 0	15	19 5	55	18 4
Oral cavity	1	0 5			1	0 3
Small intestine			1	1 3	1	0 3
Liver	54	24 3	29	37 7	83	27 8
Pancreas	2	0 9	2	2 6	4	1 3
Peritoneum	10	4 5	5	6 5	15	5 0
Myocardium			4	5 2	4	1 3
Pericardium	2	0 9	2	2 6	4	1 3
Skeleton	173	77 9	62	80 5	235	78 6
Spleen	13	5 9	5	6 5	18	6 0
Lymph nodes	192	86 5	69	89 6	261	87 3
Hypophysis	6	2 7	2	2 6	8	2 7
Thyroid	3	1 4	3	3 9	6	2 0
Adrenal	32	14 4	9	11 7	41	13 7
Kidney	3	1 4	2	2 6	5	1 7
Renal pelvis + ureter	1	0 5			1	0 3
Urinary bladder + urethra	1	0 5			1	0 3
Testis	2	0 9	3	3 9	5	1 7
Penis	1	0 5			1	0 3
Breast	15	6 8	5	6 5	20	6 7
Skin	2	0 9	1	1 3	3	1 0
Muscle	1	0 5			1	0 3

problem was later taken up *inter alia* by *Benson* (1957) and *Pribe & Ockuly* (1963) Also these authors who give a comprehensive survey of the literature, feel that lesions of the breast in these cases are most likely metastases and not primary hormone induced cancers

In cases of this type the histological appearance may be misleading unless sections from different areas of both the prostate and the breast are examined because the primary tumour and metastases may both consist of different histological components Examination of only one or a few sections may leave a false impression of two histologically different tumours Another possibility is the finding of only poorly differentiated tumour tissue at both sites i e tissue not allowing a differential diagnosis with certainty In well differentiated adenocarcinoma of the prostate the typical light glands are often helpful and were found in the breast in 14 of the 20 cases In all 20 cases, the histological picture with its absence of intraductal atypia and with no carcinoma *in situ* in the surrounding tissue argued for the lesions being metastases

Regarding oestrogen therapy as in most published series treatment had not been long and the doses had been small *Clausse & Daymas* (1951), for example described a patient with prostatic cancer who developed

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STAINING OF BETA-CELLS IN PANCREATIC ISLETS BY AN INDIRECT IMMUNOFLUORESCENCE METHOD ON BOUIN-FIXED, PARAFFIN-EMBEDDED TISSUE

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Using an indirect immunofluorescence technique a strong, selective cytoplasmic fluorescence in beta-cells was observed in *Bouin fixed*, paraffin-embedded pancreatic tissue from rats and humans This method gave positive results with anti insulin sera which were too weak to react with non fixed frozen tissue

The commonest method for the immunofluorescence technique is the preparation of fresh frozen sections in a cryostat, at times freeze drying *in vacuo* at a temperature of about —40°C

Conventional paraffin sections have not been extensively used because of the tendency of fixation and paraffin embedding to cause alterations in tissue antigens thereby preventing the binding of the specific antibody

Methods have been reported, however, where fixation—usually with formalin—and subsequent paraffin embedding have given satisfactory results

In view of the established position of the freezing method as the one best suited for immunofluorescence, those instances where fixation and paraffin embedding of the tissue seem to give just as satisfactory—or even better—results than the freezing method deserve attention

In association with an investigation of insulin bearing cells in the pancreas, we have been studying the common freezing technique together with other methods In this connection we have made the observation that the beta cells bind anti insulin-serum, even after initial fixation of the tissue in Bouin's fluid, and that this binding can be demonstrated with sera which are so weak that they do not react with snap frozen, cryostatsectioned, non fixed pancreatic tissue

TECHNIQUE

The following technique was used on normal pancreatic tissue obtained from young rats, and from humans 12 17 hours post mortem

Tissue blocks about 3 mm in thickness were divided into two equal parts One part was *snap frozen* at —70°C in isopentane in a container with a dry ice acetone freezing mixture, 6 my sections being thereafter cut in a cryostat.

The other part was *fixed* in Bouin's fluid (saturated aqueous picric acid, formalin, and

Received 3 ii 71

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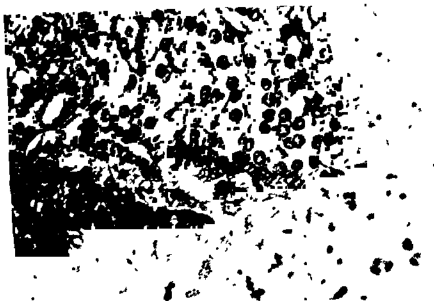


Fig 1 Part of islet of Langerhans from a rat Specific yellow-green fluorescence in beta-cells Peripherally non fluorescent alpha-cells (arrow) In the exocrine tissue non specific orange fluorescence Anti insulin stain × 330

Fig 2 Detail from the same islet as in Fig 1 Beta-cells bluish, alpha-cells reddish (arrow) Gomori's chrome alum hematoxylin phloxin stain × 530

than could be achieved with fresh freeze-dried tissue and a more precise cytologic localization was obtained No comment was made regarding this observation The phenomenon is presumably analogous to our observation

The question could be asked whether the results obtained in an immunofluorescence investigation made on Bouin-fixed tissue are the expression of an antigen-antibody reaction at all—as is supposed to be the case with frozen sections However, the usual control

acetic acid, 15 5 1) for 24 hours washed in tap water for 24 hours, in destilled water for 2 4 hours passed succesively through 70 per cent 93 per cent 99 per cent ethanol xylol and then embedded in paraffin The 6 my microtome cut sections were passed back to water for fluorescence staining

The reagents used were

a) guinea pig anti insulin serum (Mann Research)
b) fluorescein isothiocyanate labelled rabbit anti guinea pig gamma globulin (Nordic Diagnostics)
c) normal guinea pig serum

Staining of the frozen sections and the Bouin fixed tissue was carried out in the same way and in parallel

1) washing in phosphate buffered saline at pH 7 1 for 3 × 5 minutes
2) application of guinea pig anti insulin for 30 minutes
3) washing in buffered saline for 3 × 5 minutes
4) application of FITC labelled rabbit anti guinea pig gamma globulin for 30 minutes
5) washing in buffered saline for 3 × 5 minutes

The control sections included

a) unstained sections b) sections where the first layer was normal guinea pig serum c) sections on which only the fluorescent second layer was applied

After staining the sections were mounted with Fluomount and examined in a Zeiss Universal Fluorescence Microscope with a Tiyoda condensor and Osram HB 200 Mercury lamp as the source of light The primary filters were BG 38 and BG 12, or an interference filter especially adapted for fluorescein isothiocyanat (*Rygaard & Olsen* 1969) The secondary filter was type no 50 For photo micrography a Kodak High Speed Ektachrome film was used

After examination in the fluorescence micro scope the *same sections* were stained with Gomori s chrome alum hematoxylin phloxin stain (*Gomori* 1941)

RESULTS

In sections of Bouin fixed *rat* pancreas a yellow green cytoplasmic fluorescence was consistently found in the beta cells of the islets, contrasting to the non fluorescent alpha cells which in the rat pancreas are peripherally located (Figure 1)

Sections of Bouin fixed *human* pancreas also revealed fluorescence in the beta cells

In addition precise cytologic localization of the fluorescence was observed

The control sections did not reveal fluor escence in the islets

Examination of the same sections after being stained according to the method of Gomori, revealed that cells which fluoresced with the anti insulin method, stained as beta cells with bluish cytoplasm The non fluor escent cells appeared as alpha cells with reddish cytoplasm (Figure 2)

In the frozen sections of rat pancreas in this series, *no fluorescence* was found in the islets The frozen sections of human pancreas revealed a very weak *fluorescence* in a few islets

In previous experiments where a stronger anti insulin serum was used, we achieved good fluorescence in islets, both in frozen sections and in Bouin fixed sections

Bouin fixed sections from other rat organs (liver, kidney, adrenal, spleen, small intestine heart, lung and skeletal muscule) did not show signs of specific fluorescence after stain ing with anti insulin

After fixation in formalin a very weak fluorescence was found in the beta cells and after fixation in Carnoy s fluid, no fluor escence at all was seen

COMMENT

The demonstration of insulin in the islets of Langerhans by means of an immunofluor escence technique has been performed pre viously, initially by *Lacy & Davis* (1957) who made use of frozen sections

The production of fluorescence in fixed tissue by use of a serum too weak to give any reaction in frozen sections does appear sur prising In the search for previous studies of this kind an investigation by *Pierce & Midg ley* (1963) was found These investigators made use of Bouin fixed paraffin embedded human choriocarcinoma Using a fluorescent anti human chorionic gonadotropin they were able to locate chorionic gonadotropin in syncytiotrophoblastic giant cells In this way a much brighter fluorescence was observed

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QUANTITATIVE STUDIES OF THE DECAY OF LYMPHOID CELLS IN THE THYMOLYMPHATIC SYSTEM DURING INANITION AND RESTITUTION

Studies of Intact and Adrenalectomized Mice

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Studies of the decay of lymphoid cells in the thymolymphatic system have been performed on lymphoid organ suspensions from intact and adrenalectomized groups of mice subjected to varying degrees of starvation and restitution During starvation the percentage of decaying cells in the lymphoid organs increased in intact mice, but remained at normal levels in operated mice In normally fed animals the removal of their adrenal glands did *not influence lymphoid cell decay under normal conditions* It was concluded that the adrenal glands play a central role in the stress mediated decay of lymphoid cells while the adrenals under normal conditions are of no importance to lymphoid cell decay

Quantitative studies of the decay of lymphoid cells in the thymolymphatic system have been performed under a variety of experimental conditions using the nigrosin dye exclusion technique (DET) on unfixed lymphoid organ suspensions (2, 3, 5, 11, 12, 13) Recently we found that the number of decaying lymphoid cells was considerably increased after treatment with hydrocortisone (3) This observation might indicate that the adrenal glands influence the decay of lymphoid cells in lymphatic tissue especially under conditions of stress It is well known that various stress stimuli including injection of adrenal steroids result in an acute involution of the lymphatic organs (1, 6, 7, 9) and that this involution does not occur if the adrenals are removed (10)

The aim of the present study was to investigate the effects of acute starvation and restitution on the percentage of decaying lymphoid cells in the thymolymphatic system of intact mice and of mice subjected to adrenalectomy

Received 3 ii 71

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MATERIAL AND METHOD

Animals Thirty to 40 days old female mice of the inbred St/A strain were used throughout the study They were divided into two groups according to experimental procedures In the first group (35 mice) the animals were left intact In the second group (35 mice) the animals were subjected to adrenalectomy

Adrenalectomy Removal of the adrenal glands was performed on 20 to 22 days old mice under ether anaesthesia. After the operation and during

sections employed with the indirect immunofluorescence method were made, and as these controls were negative, there is no reason to suspect that an antigen antibody reaction did not take place

The negative examination of other types of tissue shows that the beta-cell staining is selective

The reason why a weak anti insulin-serum is able to bind to beta cells in Bouin-fixed tissue, and not in frozen sections is not clear, but presumably a kind of "unmasking" of determinant groups in the insulin molecule takes place under the influence of the components in Bouin's fluid

A dissolution and washing out of insulin during the staining procedure may take place, though this does not explain why stronger anti insulin sera are able to give positive results when using the same staining procedure

For the present the staining method rests upon a purely empirical basis

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thymus from intact mice during the experimental period of 8 days After three days of inanition the percentage of cell decay was significantly increased in all the organs examined (inguinal gland $p < 0.001$, $t = 5.9$, mesenteric gland $p < 0.001$, $t = 4.8$ and thymus $p < 0.001$, $t = 5.4$) After one day of restitution the cell decay in the two groups of lymph nodes examined was at normal levels The decay of thymus lymphocytes however, remained increased during the five days restitution period

Fig 2 shows the percentage of lymphoid cell decay in adrenalectomized mice during the starvation period Exposure to stress did not influence the cell decay in the mesenteric lymph node and the thymus In the inguinal lymph node the lymphoid cell decay was slightly decreased

By comparing the intact controls' with the adrenalectomized controls in Figs 1 and 2 it is evident that removal of the adrenal glands has no influence on the normal decay of lymphoid cells in the thymolymphatic system

DISCUSSION

Involution of the lymphoid organs during starvation periods is a well known phenomenon (review *Andreasen* 1943) Mice exposed to inanition show pronounced weight decrease in their lymphoid organs especially in thymus *White* & *Dougherty* (1947) showed that adrenalectomy prior to or during the starvation period inhibited a further decrease in the lymphoid organ weights They suggested that the lymphoid involution brought about by inanition in intact mice is principally mediated by adrenalocortical secretion

Treatment with steroid hormones causes a rapid decrease in lymphoid organ weights,

Figs 1 and 2 The cell decay in lymph nodes and thymus during inanition and restitution The hatched areas represent control values
Vertical bars represent ± S E M

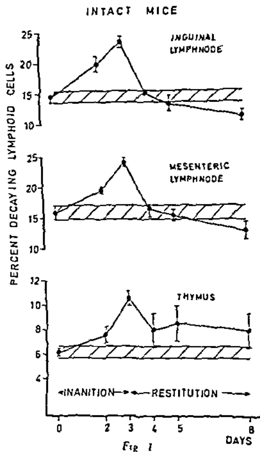


Fig 1

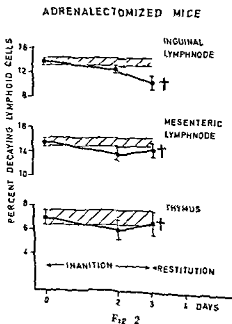


Fig 2

the experimental period the operated mice were kept in an incubator at a temperature of 26°C and were given a supplement of 1 per cent NaCl in the drinking water The presence of accessory adrenal tissue in the St/A strain was tested as follows Fifteen female mice were adrenalectomized and kept at room temperature and given ordinary drinking water All these animals died within 4 days We therefore assumed that the St/A female mouse has no accessory adrenal tissue

TABLE 1 *Experimental Design of the Experiments*

Number of animals	Adrenal ectomized	Days of starvation	Days of restitution
10	—	0	—
10	+	0	—
5	—	2	0
5	—	3	0
5	-	3	1
5	—	3	2
5	—	3	5
5	+	2	0
5	+	3	0
5	+	3	*
5	+	3	*
5	+	3	*

* These groups of mice died within the first 24 hours of restitution

Experimental design Groups of intact and adrenalectomized mice were subjected to varying degrees of starvation and restitution as indicated in Table 1 The fasted animals were kept in individual cages with screen bottoms to prevent coprophagy During the experimental period groups of mice were weighed and killed The thymus, spleen, mesenteric and inguinal lymph nodes were removed and weighed Single cell suspensions of the various lymphoid organs were made and nigrosin dye exclusion tests performed as previously described (2) Relative weights of thymus and spleen from each group of mice were calculated and expressed in terms of mg organ pr g mouse

RESULTS

Effect of starvation on the weight of spleen and thymus The weight changes during the period of starvation correspond to previous reports (1, 10) It appears from Table 2 that the decrease in relative weights of spleen and thymus of intact mice was reduced to about 50 per cent after three days of inanition The weight of the spleen increased to about normal values after five days of restitution while the thymus weight remained reduced during this period It also appears from Table 2 that no changes in lymphoid organ weights occurred during the period of starvation in the adrenalectomized groups of mice These animals, however, did not recover after the stress period

Effect of starvation on the lymphoid cell decay The results of nigrosin dye exclusion test performed on lymphoid organ suspensions correspond qualitatively to previous works (2, 3) Thus, the non-viable cells are easily recognized as dark blue, homogenous, 15 to 30 microns large cells lacking all nuclear and cytoplasmic features

Fig 1 shows the percentage of decaying lymphoid cells in the lymph nodes and the

TABLE 2 *The Relative Weight of Spleen and Thymus during Inanition and Restitution*

		Starvation period			Restitution period		
Experimental days		0	2	3	4	5	8
Intact mice	Thymus	4 1±0 3*	3 6±0 1	2 1±0 1	2 5±0 2	2 4±0 1	2 4±0 2
	Spleen	7 0±0 3	4 0±0 3	3 5±0 1	3 7±0 2	3 7±0 2	5 8±0 2
Adrenalectomized mice	Thymus	4 1±0 2	4 0±0 3	4 5±0 4	†§		
	Spleen	6 6±0 3	5 9±0 2	5 2±0 6			

* Relative organ weight ± S E M (mg organ/g mouse)
§ Adrenalectomized mice died within the first 24 hours of restitution

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COMPLICATIONS FOLLOWING EXTERNAL CARDIAC MASSAGE, WITH SPECIAL EMPHASIS ON CEREBRAL EMBOLISM

Review of Literature together with a Case Report

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This article deals with a review of the relevant literature on the theme Complications to external cardiac massage A special interest is taken in the possible neuropathological implications Since the original article by *Kouwenhoven et al* appeared in 1960, detailed observations with adequate pathological anatomical documentation concerning embolism to the central nervous system as a consequence of the performed external cardiac massage are rare in the literature A case of cerebellar embolism is reported in which external cardiac massage had been performed immediately prior to death, the relevant neuropathological techniques are emphasized

With the exception of fat and bone marrow embolism, embolism involving the central nervous system as a consequence of external cardiac massage has, to the author's knowledge not before been published in literature The reports on the well known high frequency of symptoms from the central nervous system after external cardiac massage represent the basis for this article which deals with a review of the relevant literature concerning complications to external cardiac massage, with special emphasis on the neuropathological implications

Since *Kouwenhoven and co workers* in 1960 published a technique for external cardiac massage (27) it has found wide employment and is by now established as a routine procedure in resuscitation In the Sixties, an increasing number of complications was found to be involved in this kind of treatment In the following a series of relevant papers is reviewed and quoted systematically according to the nature of the lesions which have all been regarded to be consequences of external cardiac massage Several of these lesions are often found in one and the same patient Costal or sternal fractures are commonly seen in daily clinical practice or at autopsy and represent the ones most often reported (4, 7, 8, 14, 15, 18, 20, 22, 23, 24, 26, 38, 46, 48, 53) In some cases "costochondral separations" are found (23, 42) Among the commonly reported lesions are Fat embolism (4, 6, 7, 14, 15, 20, 22, 26, 29, 46, 56), bone marrow embolism (4, 6, 7, 14, 15, 20, 23, 24, 29, 38, 48, 56, 57), cardiac lesions and haemopericardium (1, 3, 4, 5, 20, 23, 24, 26, 38, 46) and hepatic lesions (4, 8,

Received 26 ii 71

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especially in the thymus (3, 6, 7, 8, 9). Recent studies have shown that a single injection of hydrocortisone causes an increase in the lymphoid cells decaying in the various lymphoid organs and in the blood (3, 4). The weight changes of thymus and spleen observed in the present study correspond to previous studies on intact and adrenalectomized mice (10), and confirm that the stress induced lymphoid involution implies an intact adrenalocortical function.

The present study also indicates that the adrenal glands are of great importance to the increase of decaying cells in various lymphoid organs during the starvation period. Thus, the cell decay was not increased in the thymus and lymph nodes of the adrenalectomized group, whereas the intact mice showed a significantly increased percentage of decaying cells in the same period. As might be expected from cortisol treatment (3,9) and starvation experiments (1,10), thymus was the organ that was most sensitive to stress as judged by the number of decaying thymocytes. During the period of restitution the lymphoid cell decay decreases rapidly as one might expect from previous studies on stress induced changes in lymphoid organ weights (1,10). After 24 hours of restitution the lymphoid cell decay did not differ significantly from control values.

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Fig. 1. Cross section of cerebellar vessel. Sheets of cylindrical and planocellular epithelium, clumps of bacteria and possibly negative images of fat or air are seen within the lumen. (Periodic acid Schiff × 500.)

variable demyelinization were found in all specimens except the motor roots. Neither pseudocalcinosis in the basal ganglia nor focal changes were found. All these elements are characteristic findings in the neuropathological substrate which is found in long term diabetes—as described in detail by *Reske-Nielsen et al.* (39) and *Reske-Nielsen & Lundbæk* (40). No signs of embolism could be demonstrated with certainty in sections from the above mentioned locations—with the exception of cerebellum. In the area around and near to the dentate nucleus many different structures were found inside the vessels. Several sheets of cylindrical or plano-cellular epithelium and scattered cells from these tissues, small bits of alveolar walls, clusters of bacteria and fungi together with other kinds of endobronchial material were found. In some places sharply demarcated bubbles were seen which presumably were the negative images of fat or air embolism. Special staining for mucin was negative and bone marrow was not demonstrated with certainty. None of these elements were found outside the vessels, and they were present in several sections from the same specimen. The vessel walls were intact, and there were no signs of acute or chronic inflammation (Figs. 1, 2, 3).

DISCUSSION AND CONCLUSION

The literature on the above mentioned complications to external cardiac massage is complex and lack of important pathological anatomical evidence makes often the evaluation of the presented information difficult. A few authors have mentioned important, practical procedures to be applied in the neuropathological approach to some of the events to occur in the central nervous system and at other sites as a consequence of external cardiac massage.

Ribbert (1894) was the first to describe fat embolism after fracture of bones (41), and only a few years later, *Lengemann & Lubarsch* more detailed outlined the relation between fat embolism and fractures (30). It is important to note that fat embolism or bone marrow embolism may occur even in the absence of demonstrable signs of fractures (20, 22, 31, 56, 57). The explanation of

18, 23, 26, 34, 38, 45, 48, 50, 53) The more rarely reported lesions include Haemothorax (4, 8, 18, 38, 46), haemoperitoneum—either from lesions in the liver or from torn mesenteric vessels (4, 8, 38, 48, 53), pneumothorax (8, 23, 24, 26, 38, 46), splenic lesions (8, 23, 26, 38) and gastric lesions (2, 12, 33, 48, 54) Occasionally other kinds of complications have been reported Rupture of aorta (35), subcutaneous emphysema (24, 38), mediastinal emphysema (26), aspiration (46), retroperitoneal haematoma (8) and a ruptured diaphragm (48) The incidence of fractures tends to increase with age (18, 38), and there may be an accumulation in children (38) One author has found the frequency of acute myocardial infarction to be significantly higher in the externally massaged patients than in non-massaged patients (46) In at least some of the cases with cardiac lesions and haemopericardium, contributory causes—such as intracardial injections, anticoagulants and trans-thoracic pacemakers—may have been present Some of the lesions to the liver are often very severe, and this has especially been found to be true in children (51)

Cerebral embolism as a consequence of external cardiac massage has so far only been reported in detail by *Jackson & Greendyke* (22) They found significant numbers of cerebral fat emboli in 4 out of 16 patients These emboli were especially located around the internal capsule These authors used frozen sections which were stained for fat with Oil-red-O, and they did not mention bone marrow or other types of emboli

The following case presented histological evidence of cerebral embolism of different nature Embolization was found during examination of the brain from a patient who received external cardiac massage just prior to death

CASE REPORT

A 48 year old woman (Jr No 21 11 02 A nc) had for several years had a tendency to sudden occurrences of oedemata especially involving the face, and often associated with headache She had earlier been treated with Thyreoidine on account of myxoedema, but the diagnosis was not confirmed by later investigations, and Quincke s oedema was thought to be the possible explanation In 1968 she consulted her physician who found typical symptoms of diabetes mellitus and a marked excretion of sugar in the urine She was treated throughout 3 months with Tolbutamide but as she continuously was in a poor state, she was submitted for an insulin treatment and met weekly in the out patient clinic in connection with the endocrinological unit Even so her condition continued to be poor and fluctuating, and on several occasions she was brought into hospital in coma diabeticum Late in 1970 she was brought in again—this time in an unconscious condition her respiration being unstable Clinically she was under suspicion for coronary occlusion Suddenly her heart beat ceased and respiratory arrest was manifest Immediately she had external cardiac massage combined with oxygen treatment and manual ventilation This continued for the following half hour during which period she was treated with *Furosemide*, *Pethidine*, *Sodium bicarbonate*, *Isoprenaline* and *Calcium lactate* As the only response was a single series of initial complexes in the electrocardiogram—and as no other signs of life were in evidence—treatment was terminated

Laboratory tests of blood—taken just before cardiac arrest—showed signs of hyperosmolarity and a blood sugar of 312 mg %

At Autopsy, a marked coronary sclerosis was found but any signs of acute myocardial infarction were demonstrable The sternal plate had broken and the right 11th rib was fractured

Tissue specimens were obtained from pancreas kidneys stomach, duodenum ileum, colon, skin adrenals and the thyroid gland Histological examination revealed a severe diabetic angiopathy, but signs of emboli were not noted

The brain was routinely fixed in 4 per cent neutral formalin The circle of Willis showed some arteriosclerotic plaques, and the cortex showed a mild diffuse atrophy Parts of the corpus striatum the temporal lobe, the cerebellar hemispheres with the dentate nucleus, the cervical lumbar and caudal medulla and the spinal roots were removed for histological examination In addition to the staining methods routinely used in this department in the study of such cases—haematoxylin eosin van Gieson, toluidine blue periodic acid Schiff and Weil—the following special techniques were employed Staining for mucin (mucicarmine) Gram staining for bacteria and fungi, and staining for elastin

Histological examination of the central nervous system of our patient revealed characteristic pathological changes A mild angiopathy with increase of PAS positive material in the vessel walls, diffuse, degenerative changes in the brain tissue with

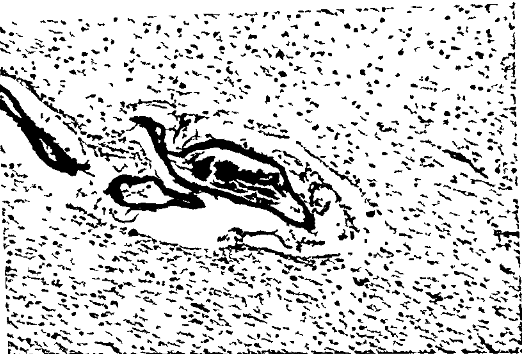


Fig 3 Cross section of cerebellar artery Small bits of alveolar walls (Periodic acid Schiff x 560)

Fat embolism to the brain has even been reported to occur after Per Abrodil myelography (25) *Chomette et al* (7) found that systemic involvement may often be quite assymptomatic

An essential problem is the frequency of fat embolism which is found at autopsy in patients in whom there is no demonstrable relation to any of the above mentioned conditions *Tedeschi et al* (52) state The identification of occasional intravascular droplets of neutral fat is relatively frequent in post mortem tissues Other authors have made the same observations in their own control groups (19 55 57) There is furthermore the danger of artificial emboli which may easily be induced by the pathologist himself at autopsy Emboli consisting of atheromatous material have been found in the brain vessels following other kinds of trauma 49 and this type of embolism might very well happen when external cardiac massage is performed in older people Tissue embolization in the brain after a traffic accident has been reported by *Gulkin* (16)

In cases where *cerebral embolism* can be suspected the plexus chorioideus should be one of the first places in which the emboli should be looked for because of its rich vascularity (44 47) In cerebellum, where metastases are often found in daily neuropathological practice observation of emboli may be difficult because cerebellum seems to be particularly prone to degenerative changes—probably due to locally altered metabolism secondary to ischaemia or drastic metabolic changes of other types (21, 36) An easy procedure is to remove the plexus in toto and transfer it to a slide glass for direct examination under the microscope (47) Where fat embolism is suspected the whole plexus may be stained directly in Sudan for a short time (47) When air embolism is suspected it is necessary to remember that it is not uncommon to see tiny columns of air in the leptomeninges of dead persons without any signs



Fig 2 Cross section of cerebellar artery Typical cylindrical epithelium close to sharply demarcated negative image Large snowball like structure of undefined material (Elastin staining x 720)

this might be microfractures and such fractures have been reported by *Tattal & Wyatt*, who demonstrated trabecular microfractures with intramedullary ecchymoses in serial sections from apparently normal ribs in which no damage could be seen by x ray examination (14) *Yoell* has reported 2 cases of bone marrow embolism following sternal puncture (58) A direct communication between the sternal marrow and the internal mammary vein which was discovered by *Pizzolato* in 1944 might play an important role in the development of fat or bone marrow embolism (37) According to the literature the percentage of patients in whom some kind of embolism in prone to develop after external cardiac massage varies from 12 per cent (5) to 100 per cent (7) and it seems as if a realistic figure should lie around 20–40 per cent in unselected materials this is consistent with the findings obtained by several authors The differences probably vary with the skill exerted by the examiner and the technique used as well as with the selection of the patients and the extent and care displayed in the search for the emboli In animal experiments *Lubarsch* produced evidence that fragments of experimentally provoked bone marrow emboli could still be found in the lungs of the animals after 6 days and it was not until after four weeks that the last signs of embolism disappeared (32) This may be an indication of the length of intervals after embolic episodes during which the evidence we are looking for still can be expected In 1968 *Tedeschi et al* (52) made a thorough review of the many causes of fat embolism Injury of soft tissue shock pancreatitis various intoxications infection with cl Welchii osteomyelitis fractures sickle cell crisis fatty nutritional cirrhosis cardiopulmonary bypass and many others Cardiac surgery has especially in earlier time been a common cause (13 19) Fat embolism after decompression sickness has been a fact known for the last decade (9 10 17 51)

—dependent on the nature of the emboli sought for

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of autolysis or any possibility of earlier air embolism In such cases no air has been found in the plexus chorioideus (47)

In the *lungs* it is necessary to obtain specimens from representative—peripheral and central—parts of the pulmonary tissue When air embolism is suspected, it is possible to clear fresh specimens directly cut from the lung with 2 per cent potassium hydroxide before examination under a stereomicroscope (43) It is however dangerously easy to produce artificial air embolism Utmost care should be observed in the preparation

It applies to *both tissues* that it is important to avoid the routine alcohol dehydration or fixation during a search for fat embolism Frozen sections in combination with specific fat staining methods may be convenient in *many cases* In some cases it is particularly necessary to search in both the pulmonary and the brain tissue In the field of forensic medicine, cerebral fat embolism without simultaneous pulmonary fat embolism suggests that the patient was alive at the moment of decapitation (28)

In the here reported patient it cannot be doubted that the performed external cardiac massage had caused damage to the pulmonary tissue and the bronchi with subsequent tearing off of small bits of epithelium with endobronchial contents and small pieces of alveolar walls which apparently had been pressed into the pulmonary veins and via the blood stream been transported to the cerebral vessels Rather curiously the embolic material could only be demonstrated with certainty in the area around the dentate nucleus but this must be regarded as a mere coincidence or as a result of the preparation The bubbles inside the vessels are possibly air or fat, but as the specimens were routinely dehydrated in increasing concentrations of alcohol this cannot be known Only massive and widespread fat embolism could be regarded as significant in this respect as it would reveal the true nature of such bubbles during the histological examination in spite of alcohol dehydration Identification of bone marrow —in particularly megakaryocytes or typical myelopoietic or erythropoietic cells—could be regarded as a proof of embolism when found inside a vessel This was not found in this case Other pathognomonic findings are—as demonstrated here—typical sheets of cylindrical epithelium of normal type and clumps of bacteria and fungi inside the vessels with no signs of acute or chronic inflammation

No doubt, the incidence of cerebral embolism following external cardiac massage is much higher than that otherwise apparent from a review of literature Occasionally systemic involvement in addition to proven pulmonary embolism has been mentioned in the relevant literature a statement which often is based on an occasional finding of some bone marrow in for example a glomérulus However—careful, systematic examination knowledge of performed external cardiac massage, of the different types of emboli and their nature and knowledge of where to seek are necessary in order to establish a diagnosis which implicates a connection between performed external cardiac massage and the actual embolic episode

It is very difficult—perhaps even impossible—to evaluate the damage of these emboli which must be regarded as iatrogenic in nature External cardiac massage is to-day rightfully regarded as a valuable tool in resuscitation but there seems to be little doubt that too violent techniques may add new complications to an already complicated situation at least in some patients Embolism can only aggravate the ischaemia which is already threatening the central nervous system because of the primary disease of these patients which led to the initiation of external cardiac massage Damage to the heart as a consequence of external cardiac massage may very well in some cases be added to the heterogenous *group of factors* which may lead to iatrogenic cardiac arrest (11)

Greater series of well investigated patients are needed in order to make a serious approach to this delicate problem and it is important to note that special procedures in neuropathology may be necessary in this work

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PRIMARY POLYCYTHAEMIA

2 Types of Chromosome Aberrations in 21 Clones Found in Bone Marrow Samples from 50 Patients

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In previous investigations the presence of clones of bone marrow cells with abnormal karyotypes were demonstrated in patients suffering from primary polycythaemia (P P) Among the first 50 patients of our series a total of 21 cases with clone formations was found comprising from 25 per cent to 100 per cent of the bone marrow cells analysed All 21 clones were found in treated patients The karyotypes of the clones are described in detail In three cases the karyotypes were aneuploid the remaining 18 clones presenting diploid karyotypes On the basis of purely morphological studies it is discussed whether the karyotypes in the 18 clones are balanced It is concluded that by the present analytical technique this question can hardly be solved In 6 cases karyotypes with a deleted F group chromosome were found, in another 2 cases, in addition to other abnormalities there was a deleted F group chromosome The specificity for P P of the deletion of an F group chromosome is discussed and it is concluded that some degree of association exists between the F-deletion and P P However it is pointed out that the F deletion has been found occasionally also in patients with other haematological disorders In 5 cases clones were found in patients with P P who died subsequently in acute leukaemia No fundamental differences between the karyotypes of these clones and those of the other clones observed in our series were demonstrated The large size of the clones shows that the clone cells must possess selective advantages but the factors determining the propagation of clones are unknown It is also uncertain whether the large clone formations found in patients with P P are in any way related to the increased incidence of leukaemia

In previous reports (16 17) we have described clone formations in bone marrow aspirate from some patients with primary polycythaemia (P P) In the first 25 patients from whom it was possible to analyse a sufficiently high number of bone marrow cells, a total of 12 clones was observed The series was divided into treated and untreated patients The group of treated patients was divided into those without myelofibrosis those with myelofibrosis and those in whom incipient transition into a leukaemic phase was possible Clone formations were found in all three groups of treated patients

The chromosomal pattern of the clones varied from one case to another, and the limited number of clones observed did not permit any conclusion as to the type and specificity of the clonal chromosomal patterns in P P An extension of the series to twice the original number, with recording of a total of 21 clones, calls for a further analysis of the chromosomal patterns

On the basis of a survey of the chromosom-

Received 27 ii 71

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TABLE 1 *Clones with Abnormal Chromosome Pattern in Bone Marrow from ³²P Treated Patients with Primary Polycythaemia*

	♂/♀	Age	Date of analysis	No of cells analysed	per cent clone cells	Karyotype	Tentative explanation
	♂	80	Jan 69	51	56	46,XY B— 16+	deletion of a B-group chromosome
	♂	62	Jan 69 March 69	60 28	73 100	46,XY,F?—	deletion of an F group chromosome
	♀	64	March 69	49	94	46,XX 1? + B— C— C— 16 + 16 + 16 +	deletion of a B- and two C-group chromosomes translocation to a chromosome No 1
	♀	83	May 69	23	83	46,XX 2— 3 + Bq +	deletion of a chromosome No 2 translocation to a B-group chromosome
	♀	81	June 69	51	32	46,XX F?—	deletion of an F-group chromosome
	♂	58	June 4th 69 June 25th 69	12 63	35	47,XY C +	trisomy C
g	♀	64	June 69	26	61	46,XX F?—	deletion of an F group chromosome
h	♀	78	June 69	12	58	46,XX C— D + ,Dq—,F?—	deletion of a C- D- and F group chromosome
	♀	73	Oct 69	100	91	46,XX 2 + C—	translocation to a C-group chromosome
j	♂	60	Oct 69	53	87	46,XY,F?—	deletion of an F-group chromosome
k	♂	72	Oct 69	79	100	46 XY Dq—	deletion of a D group chromosome
l	♂	73	Oct 69 Nov 70	100 40	58 80	46,XY B + C— C—,D +	translocation between two C group chromosomes
m	♀	80	Nov 69	92	93	46,XX 1? +	translocation to a chromosome No 1
n	♀	62	Feb 70	30	63	46,XX C—,D + Gs +	translocation from a C to the satellites of a G group chromosome
o	♀	70	Feb 70	53	25	46,XX,Dq— Ep +	translocation from a D to an E-group chromosome
p	♂	60	Feb 70 April 70	23 53	91 85	45,XY 3— Cr	monosomy 3 and a ring C chromosome
q	♂	40	March 70	67	84	46,XY,F?—	deletion of an F group chromosome
r	♂	73	May 70	12	42	46,XY F?—	deletion of an F group chromosome
s	♂	67	March 70	24	54	47,XY C + F?—	trisomy C and deletion of an F-group chromosome
t	♀	59	May 70	26	31	46,XX E— G +	deletion of an E-group chromosome
u	♀	73	May 70	15	40	46,XX Gq—	deletion of a G group chromosome

Another limitation which may play a role is our present definition of a clone As was the case in our previous studies into radiation induced chromosome aberrations i e (13) we use the designation clone formation only when the following criteria are satisfied

a) At least three cells having completely identical structural chromosome aberrations must be found b) No other chromosome aberrations must be present in these cells — If clones with numerical aberrations are observed, the extra or missing chromosomes must be identical and in cases of hypodiploid karyotypes a higher number of clone cells

al pattern in all 21 clones, we wish to comment in particular on the following problems

1 Number and size of clones
2 Frequency of aneuploid/diploid karyotypes
3 The possibility of balanced karyotypes
4 The specificity of the F deletion for P P
5 The karyotypes of clones in patients dead in leukaemia
6 The pathogenesis of the clones

A further review of clinical data and studies of the significance of the clinical condition and the treatment for the development of the clones will be reported on in a subsequent paper (18)

MATERIAL AND METHODS

The material comprises 50 patients from the Radiumhemmet, Stockholm The patients are selected, the aim being to have 4 groups represented untreated patients, treated patients without signs of myelofibrosis, treated patients with myelofibrosis and, finally, treated patients in whom incipient transition into a leukaemic phase was suspected clinically

In addition to the clinical examination which included haematological data histological and cytological examinations of the bone marrow were carried out at the time when bone marrow aspirates and samples of peripheral blood were obtained for chromosome analysis The blood and bone marrow specimens were immediately transferred to the Chromosome Laboratory in Copenhagen where the preparation was started not later than four hours after the samples were drawn

In some of the patients the analyses were repeated The aim was to analyse between 50 and 100 cells from both blood and bone marrow from each patient In a few patients no more than between 10 and 20 analysable cells were found in the bone marrow aspirate If less than this quantity of cells from the bone marrow was analysed the patient concerned was excluded from the series

The bone marrow specimens for chromosome analysis were prepared using a modification of the direct method of *Tjio & Whang* (11) The blood samples were analysed after 48 hours culture and in other respects by the method of *Moorhead et al* (9)

Complete chromosome analyses were made of each individual cell and many of the cells were photographed

RESULTS

Of the bone marrow specimens from the 50 patients (surveyed as at December 1st, 1970), 21 presented large clone formations These 21 clones were all found in treated patients

Table 1 contains a survey of the clone formations

The number of clones found in the various groups of the series are as follows

8 untreated patients 0 clones,

23 treated patients without myelofibrosis 7 clones (f, i, j l, m, n, o),

11 treated patients with myelofibrosis 9 clones (e, g, h, k, q, r, s t, u),

8 treated patients in whom incipient transition into a leukaemic phase was possible 5 clones (a, b, c, d, p)

DISCUSSION

The present report contains data concerning only bone marrow studies In the peripheral blood only few clone cells were observed and only in a limited number of patients In a previous study (15) concerning the first 25 patients, the correlation between the typical radiation induced chromosome aberrations and the total dose of ^{32}P was investigated Up till now, it has not been possible to demonstrate the existence of a positive correlation between dose and frequency of aberration

1 *Number and Size of Clones*

The present method of chromosome analysis must be considered a crude morphological test with limited potentialities, even when the analysis is carried out meticulously In particular, it will be difficult to record minor abnormalities in the C group chromosomes and consequently, it must be expected that the number of clones recorded is presumably lower than that actually present Most likely the recent methods employing fluorescence staining of the chromosomes, i e (19), whereby each individual chromosome can be characterized, will enable us to observe clones in the samples from a greater number of the patients

aberrations in human cells are well known Hitherto the principal rule has been (13) that cells with radiation induced chromosome aberrations which are able to continue reproduction carry the so-called stable forms of aberrations and that they have balanced karyotypes, i e, karyotypes in which no chromosome material is lost and no excess material is present Only re arrangement of material has occurred between the various chromosomes

Up till now it has been possible to base a study into the question as to whether the karyotypes of the clones observed are balanced, only on purely morphological studies and, with the present analytical technique, this is not satisfactory

Table 1 presents the aberration pattern of the clones described in accordance with the *Chicago nomenclature* (1), and a tentative explanation is advanced, whereas no statement is made as to whether the karyotypes are believed to be balanced

The three aneuploid clones surveyed above, i e f p, s are of course not balanced

The clones l n o, d are considered to be balanced

It is difficult to evaluate the clones b, e, g, j q r in which a deleted F group chromosome is the only recognizable aberration, because the material concerned is so limited

Also the clones a h k t u present apparently quite minor deletions and also in these cases the assessment is uncertain

Clone c has a karyotype in which many chromosomes are involved The karyotype may be balanced but the assessment is very uncertain

The clones i and m seem to have karyotypes with very small quantities of excess material In these cases the assessment is also uncertain

If the three aneuploid clones are disregarded it must be concluded that, on a purely morphological basis it is not possible to decide for certain whether the clones are balanced or not It is supposed that the solution will be easier when the study of the patterns of the individual chromosomes will now be made possible with the introduction of the fluorescence staining method (11) However, we have not yet made more comprehensive tests employing this technique If the clones are balanced, it supports the theory that they originate primarily from radiation injured cells On the other hand, our present knowledge does not allow any conclusions to be drawn as to whether or not the clone cells with unbalanced karyotypes have a different genesis

In one treated patient only, *Lawler et al* (5) observed a clone with an obvious reciprocal translocation

Fig 1 (clone n) presents an example of a karyotype which is supposed to be balanced

4 *The Specificity of the F deletion for P P*

It appears from Table 1 that, in 6 cases, a deleted F group chromosome was the only aberration found in the clones, in another two cases this finding was associated with other abnormalities

In the reports on the series from the Royal Marsden Hospital (4, 5, 7, 8), the several cases of clones with deleted F group chromosomes were mentioned at an early date It was emphasized that some degree of association might exist between this abnormality and P P Since similar conditions have now been found in another series, this important result must be thought to be definitely verified

The appearance of the deleted F group chromosome may vary slightly In many cases the only finding seems to be the deletion of a very small quantity of material from one arm, in other cases a somewhat greater quantity is deleted (Fig 2), and in some cases the entire chromosome appears to be slightly reduced in size

It is important to clarify the specificity of the F deletion for P P and, in this connection, also the question as to whether it might be induced by the treatment The most recent results obtained in the Royal Marsden Hospital and made available to the author (*Millard*, personal communication (6)) show that the F-deletion has now been found in 15 treated patients Thirteen patients had

and analysed cells is required, although no definite numbers have been established

It appears from Table 1 that only one clone was detected in each patient and that the clone cells comprised a large proportion of the cells analysed In one patient the clone included 25 per cent, in four 30 50 per cent and in 15, more than 50 per cent of the bone marrow cells analysed No correlation was found between the size of the clones and the various types of chromosomal pattern presented by the clones The implication of the large proportion of clone cells will be discussed later together with the pathogenesis of the clones

2 *Frequency of Aneuploid/Diploid Karyotypes*

It will be seen from Table 1 that clones with aneuploid karyotypes were found in 3 patients only, i e, the clones f p s, all other clones had diploid karyotypes

Clone f was found in a patient who had suffered from polycythaemia *for 13 years and* who presented a hyperplastic bone marrow without fibrosis Incipient transition into a leukaemic phase was not suspected The clone *comprised about 35 per cent of the cells and* the karyotype contained 47 chromosomes with an extra C group chromosome

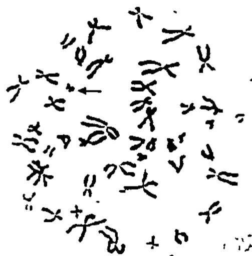
Clone p was found in a patient who had had polycythaemia for 9 years prior to the first chromosome analysis *His* bone marrow presented pronounced fibrosis and sclerosis but contained few stem cells only Incipient transition into a leukaemic phase was suspected clinically —Three chromosome analyses on the bone marrow were made The first analysis failed partly, because the quality of the specimens allowed analysis of a few cells only, but by screening it was found that the majority of the cells in mitosis contained a ring chromosome Two subsequent analyses in February 1970 and April 1970 revealed a clone with 45 chromosomes a ring chromosome, presumably originating from a missing C group chromosome and lack of a number 3 chromosome Several tetraploid cells were observed with 90 chromosomes and two rings The patient died in May 1970 in acute leukaemia

Clone s was found in a patient who had suffered from polycythaemia for 11 years The bone marrow presented pronounced fibrosis and sclerosis Transition into a leukaemic phase was *not suspected The clone* was easily recognizable because of the deleted F group chromosome and a total of 7 out of 24 *clone cells analysed had 47 chromosomes* with an extra C group chromosome In the remaining cells the chromosome numbers *varied greatly ranging from 37 to 94*

Hence an aneuploid clone was observed in each of the three groups of treated patients In none of the cases did we reveal an evolution in the formation of the clones as described by *Lawler et al* (5) in a few of their patients Their series from the Royal Marsden Hospital which forms the basis of a number of reports (4 5 7 8) seems to be the only material which lends itself to a comparison with our patients These studies have now been extended to comprise a total of 79 patients with P P (5) Aneuploidy and apparently aneuploid clones were detected in 5 out of 33 untreated patients Our series includes 8 untreated patients only and we have not observed clones in any of them No conclusions can be drawn on the basis of these figures Furthermore *Lawler et al* observed aneuploidy and aneuploid clones occasionally *with changes in* the chromosome number during the course of disease in almost all patients who had developed leukaemia and *occasionally in* treated patients without leukaemia

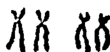
3 *The Possibility of Balanced Karyotypes*

If the etiological mechanisms underlying the formation of clones are to be investigated the primary basis must be a *detailed analysis* of the karyotypes of the clone cells In the present study of ³ *P* treated patients *it is only* natural to investigate whether the karyotypes of the clones are of such a nature that the clones might originate primarily from one single radiation injured cell The various types of radiation induced chromosome

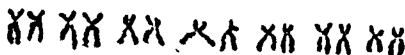




1 —— 3



4 — 5



6 —— 12



13 — 15



16



17 — 18



19 — 20



21 — 22



X X

Fig 2 Clone g Karyotype of clone cell from bone marrow from a ^{32}P treated patient with primary polycythaemia Deletion of an F-group chromosome

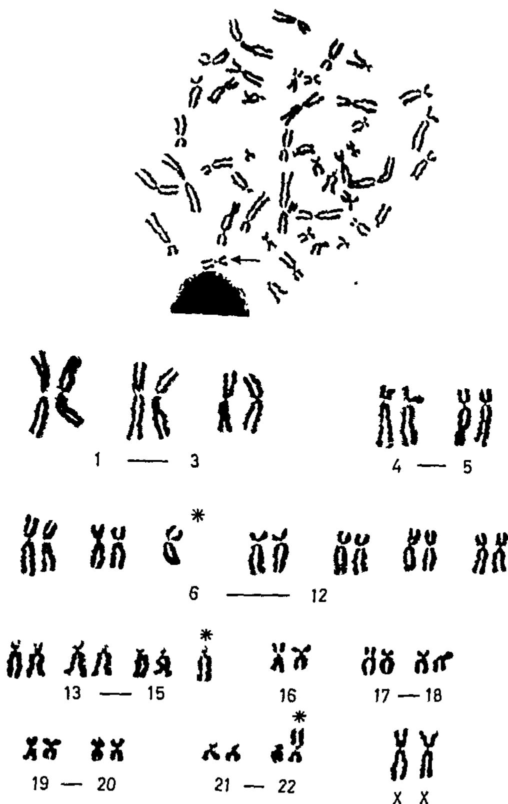
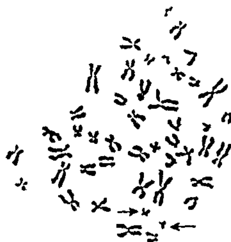
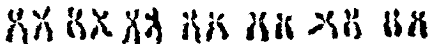


Fig 1 Clone II Karyotype of clone cell from bone marrow from a ^{3} *P* treated patient with primary polycythaemia Translocation from a C to the satellites of a G group chromosome Presumably balanced karyotype





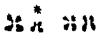
















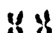


Fig 3 Karyotype of clone cell from bone marrow from a patient with chronic myeloid leukaemia Philadelphia-chromosome and deletion of an F group chromosome

been given ^{32}P, in one case splenic irradiation had been administered, and one patient had received busulphan treatment only. Furthermore, the aberration was observed in an additional patient who was treated with venesectio only. Therefore, it is believed that the F-deletion is not necessarily induced by radiation.—As stated above, in our series only those patients who had been treated with ^{32}P presented F-deletion.

It is of considerable interest that *de Grouchy et al.* (3) observed a similarly deleted F group chromosome in some patients with sideroblast anaemia and in some patients with subacute myeloid leukaemia. In our laboratory, in one case of Ph^1 positive chronic myeloid leukaemia, we observed an F group deletion in between 54 per cent and 80 per cent of the Ph^1 positive cells by repeated analyses (Fig. 3). These observations appear to be of great theoretical interest although, at present, we are unable to determine the significance of the F deletion in these various haematological disorders. Further studies into these conditions seem to be indicated before well established theories can be advanced. However, it seems justifiable to conclude that most likely the F deletion is especially frequent in P.P., but not specific for this disorder.

5. The Karyotypes of Clones in Patients Dead in Leukaemia

As stated above our series includes chromosome analyses on 8 patients in whom incipient transition into a leukaemic phase was possible. All these patients have later died. Seven of them died in acute leukaemia, one died from pneumonia, but 2 per cent pro-myelocytes were found in the peripheral blood and a number of pathologic stem cells in the bone marrow. Consequently, it must be presumed that also in this case the early phase of leukaemia has influenced the course of disease. Finally, one patient died from myelomatosis. When transition into a leukaemic phase occurs in patients with polycythaemia, the type of leukaemia will always be acute or subacute. Of course, the possibility cannot be excluded that these patients might develop Ph^1 positive chronic myeloid leukaemia, but at least that it occurs very seldom. On the other hand, there is no doubt that a correlation exists between polycythaemia and myelomatosis, and this is probably rather unknown. At the Radiumhemmet in Stockholm, 6 patients with P.P. have been encountered who also suffered from, or later developed myelomatosis. Three of these cases have been published (2).

As previously mentioned, clones were observed in five of the eight patients who died from or showing signs of acute leukaemia, i.e., clones a, b, c, d, p. The patient who died from myelomatosis carried the clone i. Hence no clone formations were found in three of the patients.—*Lawler et al.* (5) state that no case of acute leukaemia following P.P. with normal chromosomes has been recorded. Presumably, this statement is based on the fact that chromosome analyses have been published occasionally only in these patients. Furthermore, the abnormalities found in the karyotypes of the clones are often so minute that cases are bound to occur, in which the aberrations are too small to be recognized by our present analytical technique and consequently, if only for this reason, it cannot be supposed that all these patients should present chromosome abnormalities.

Details concerning the six clones (a, b, c, d, p, i) can be derived from Table 1 and the survey on page 517. They are all large clones but when considered collectively no differences appear to exist in the karyotypes in this group of clones and the remaining clones found in the series, since all types seem to be represented. It should be mentioned specifically that only one aneuploid clone (p) was observed. In the survey published by *Lawler et al.*, aneuploid conditions were observed in seven or eight of their nine patients with leukaemia. This difference may be due to the fact that the latter authors were able to make chromosome analyses later in the course of disease than we have been able to do in several of our cases.

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6. *The Pathogenesis of the Clones*

It has already been stated that clone formations were not observed in the 8 untreated patients included in our series of 50 patients. Neither have clone formations been found in the 8 patients who had received Myleran only. All the patients with clones had been treated with ^{32}P, with the exception of one (clone k) who had received splenic irradiation and Myleran. These findings speak strongly in favour of the etiological significance of ^{32}P-therapy for the development of the clones, see also the statements above regarding the karyotypes of the clones. However, it should be pointed out that, in the somewhat more comprehensive series from the Royal Marsden Hospital which, inter alia, differs from our sample in that it contains a higher number of untreated cases, a few aneuploid clones have been observed both in untreated patients and in patients treated with Myleran only.

It must be assumed that the clones with the often very specific chromosomal patterns must be derived primarily from one abnormal cell. The large size of the clones which often occurs, see Table 1, proves that the clone cells possess pronounced selective advantages. In other words, these cells are more or less "insensitive" to the normal homeostatic control of the formation of bone marrow cells. Our knowledge of these regulatory mechanisms is very limited and, up till now, as far as we know, no studies have been reported which offer any explanation of the propagation of the clones found in these patients. It is of outstanding interest that it has been observed that during treatment cells with an abnormal chromosomal pattern may be suppressed, so that the more normal haematologic picture obtained is associated with a decrease in the abnormal cells in the bone marrow (5).

The fact that many of these patients have a high proportion of cells with an abnormal chromosomal pattern in their bone marrow, poses the question as to whether this might be a contributory cause of the high frequency of leukaemia. It is a well-known fact that many disorders exist, in connection with which patients with specific chromosome abnormalities present an increased tendency to development of leukaemia, i.e., *Visfeldt* (14), and it has also, by *in vitro* experiments been possible to demonstrate an accelerated rate of SV 40-induced malignant transformation in cultivated cells from some patients presenting a high incidence of chromosome abnormalities (10, 12). Therefore, the possibility cannot be excluded that the clone cells represent populations which might have an increased tendency to malignant transformation, so that a connection may exist between the chromosomally abnormal bone marrow cells and the increased tendency to leukaemia in patients with P.P.

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RESULTS AND DISCUSSION

Fig 1 presents graphically the number of newly diagnosed cases of deaths from lung cancer in Sweden during the period 1959–1966

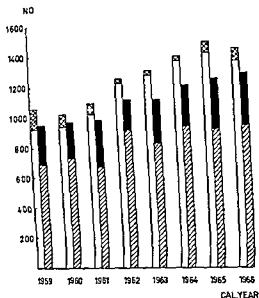


Fig 1 Annual number of new cases of and deaths from lung cancer in Sweden in 1959–1966

- Number of new cases of lung cancer specified as primary
- Number of deaths from lung cancer specified as primary
- Number of new cases of lung cancer unspecified as to whether primary or not
- Number of deaths from lung cancer unspecified as to whether primary or not

Both the number of cases of specified primary lung cancer and the number of cases of lung cancer unspecified as to whether primary or not are given separately for men and women The frequencies of unspecified lung cancer cases are very different in the morbidity and mortality data It should be pointed out that the former figures pertain to the incidence of primary neoplasms and the latter to the underlying cause of death where the chain of events is not always known During the years 1965 and 1966, there were 141 deaths where lung cancer was the contributory cause of death and half of these cases were unspecified as to whether primary or not Fluctuations during 1960–64 in the mortality from primary lung cancer indicate inconsistencies in classification and coding practices The number of unspecified cases of lung cancer in the incidence series declined during the middle of the period, probably for similar reasons The cases of lung cancer not otherwise specified (Int List No 163) should be included in the comparison between lung cancer incidence and mortality The differences in frequency of such unspecified cases are surprisingly large The unspecified cases constitute 26 per cent of the mortality series against only 5 per cent of the morbidity series This is chiefly because the information in death certificates often is very insufficient The incidence of lung cancer specified as primary or not otherwise specified (Int List No 162 1 and 163) is higher than the corresponding mortality and the difference increases towards the end of the 8-year period There are several conceivable explanations of this

1 Losses' in the morbidity series, in the form of non reported, known cases, was probably larger at the beginning than at the end of the period An investigation of completeness and accuracy in reporting of known lung cancer cases from a limited region of Sweden has however, not given any support for such an assumption (*Larsson* 1970 a)

2 A true increase in incidence, with unchanged mortality pattern causes some divergence of the incidence and mortality curves. The mortality pattern probably changes somewhat from year to year depending on structural changes in the lung cancer population with an increasing proportion of older persons (*Larsson* 1970 b) Thus at least some of this effect of the increase in incidence is neutralized

3 The therapeutic effect is a plausible and more attractive explanation In a very high percentage of cases the natural history of this disease from the time of diagnosis is alarmingly short Thus, the average duration of the surgically untreated cases, constituting 75 per cent of the unselected series of 998

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COMPARISON OF LUNG CANCER MORBIDITY AND MORTALITY IN SWEDEN 1959–66

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A comparison between the lung cancer incidence and mortality in Sweden during the 8 year period 1959–66 is presented. The incidence was higher than the mortality and the difference increased towards the end of the period. Conceivable explanations are discussed. The mortality attained 86 per cent of the incidence in the 4 year period 1963–66. The difference was especially pronounced in the age groups under 70 years. Analysis of an unselected series of patients with primary lung cancer showed that it could probably not be attributed to divergencies between patient categories under and over 70 years in the distributions of histological types but very likely to an effect of successful surgery in the lower age groups.

Morbidity and mortality statistics in lung cancer do not seem to have been compared. Statistical and epidemiological studies in many countries have largely been based on mortality data due to lack of satisfactory morbidity data. Incidence is the most precise measure of risk of a given disease in a given population. Death and incidence rates are most closely parallel for neoplastic diseases of brief duration between diagnosis and death, where survival rates have not changed over the years. This is so in lung cancer (*Schottenfeld & Houde* 1966). Because of its high fatality, i.e. the number of persons dying from it is nearly equal to the number affected, mortality statistics provide a reasonable estimate of the incidence (*Doll* 1953). Five-year survival rate of patients with this disease is around 5 per cent (*Bignall* 1958, *Eisenberg et al.* 1964) and has not substantially improved during recent decades (*Boucot et al.* 1967). This study compares lung cancer incidence and mortality in Sweden 1959–66 and analyses differences.

MATERIAL

Data on the total Swedish population during the period 1959–1966 were utilized for comparing the mortality and morbidity statistics in lung cancer. Primary lung cancer is recorded under code number 162.1 and lung cancer, unspecified as to whether primary or not, under code number 163 as recommended by *WHO 1957* (the Seventh Revision of the International Statistical Classification of Diseases, Injuries, and Causes of Death). The incidence and mortality in both these categories are shown. The mortality figures were taken from the annual reports of *The Central Bureau of Statistics*. The incidence data were obtained from *The Cancer Registry of Sweden*. The number of cases under code numbers 162.1 and 163 in different age groups during the years 1963–1966 were compiled for a more detailed analysis of incidence and mortality. Frequency of resection, 5 year survival rate and histological type distribution by age were based on an unselected series of 998 cases of primary lung cancer diagnosed in two provinces of Sweden 1959–65.

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TABLE 3 *Number of New Cases of and Deaths from Lung Cancer (Int List Nos 162 1 and 163) by Age in Sweden 1963 1966, and the Difference Between Incidence and Mortality*

Age years	New cases	Deaths	Difference No	Difference %
0–39	54	48	6	11
40–49	288	233	55	19
50–59	1053	825	228	22
60–69	2161	1833	328	15
70–79	1602	1462	140	9
80–	431	410	21	5
All ages	5589	4811	778	14

and mortality are statistically unreliable in these small age groups, and partly because the few cases (18 in 1965) in which lung cancer was recorded only as the contributory cause of death are probably concentrated in these age groups In absolute terms, the difference in incidence and mortality in women over 70 years was only 26 in 1965 It has been stated that mortality statistics provide a reasonable estimate of the incidence of lung cancer because of its high fatality During the 8 year period 1959–1966 no increase in lung cancer was observed among men under 65 (*Larsson* 1970 b) and the mortality pattern in these age groups has probably remained unchanged Therefore it was considered worthwhile directly to compare the recorded incidence and mortality in absolute numbers in various age groups during the 4 year period 1963–1966 The result is presented in Table 3 It shows the number of newly diagnosed cases and of deaths by age groups, and the differences in absolute numbers and as percentages of the incidence Had the mortality during this period been used for estimating the incidence the result would have been a deficit of for example, 22 per cent in the age group of 50–59 years and 14 per cent totally If cases where lung cancer was only a contributory cause of death are considered the difference in lung cancer frequency between the morbidity and mortality data is reduced The number of such cases is available for the years 1965 and 1966 The total difference between the incidence and mortality during these two years was 14 per cent If the cases where lung cancer is listed as a contributory cause also are included in the mortality figures, the difference expressed in per cent of the incidence will be reduced to 9 per cent

The group of unspecified cases (Int List 163) is very large in the mortality series and perhaps not homogeneous It has not been analyzed in this connection, but upon examination would probably be reduced On the other hand, the Cancer Registry has not utilized information provided by death certificates for registration purposes, and some cases of lung cancer known by death certificate only are not included The shown difference between the number of cases listed by the Registry and mortality statistics thus seems to be on the small side

Mortality rates are considered adequate for studies of comparative incidence in countries where the accuracy of death certification is high, and for cancers which have a high fatality rate (*Doll et al* 1966) Several authors have discussed the inaccuracy inherent in the certification of causes of death (*Gilliam* 1955, *Registrar General* 1957, *Pedersen* & *Magnus* 1959, *Willis* 1961, *Bolander* 1969, *Rosenblatt et al* 1969, etc.) and the present study showed that cancer registration gives considerably more reliable information on lung cancer incidence than the mortality statistics especially in low age groups Despite the documented high mortality, it is not an accurate estimate of the true risk of this disease in the population

CONCLUSIONS

1 It became evident from this investigation that the morbidity statistics gave a considerably more accurate answer about the true incidence than the mortality statistics

2 The difference between recorded incidence and mortality seemed to be the result of effective therapy in the lower age groups

3 Morbidity data are preferable to mor-

cases of primary lung cancer, was barely 4 months. Nineteen per cent lived for 14 months, on an average, after surgery. The remainder of the patients, i.e. 6 per cent, who were also operated upon, all lived for more than 5 years (*Larsson* 1970 c). The difference between the incidence and the mortality is therefore a coarse measure of the effect of therapy, but it depends not only on those that were successfully treated, but even on single untreated cases with surprisingly protracted courses and on those that suffered from lung cancer but died from other diseases. In such cases, lung cancer is recorded only as a contributory cause of death in the mortality tables. The number of such cases was 66 in 1965, while the difference between the incidence and the mortality that year was 246.

The age-specific incidence and mortality rates of lung cancer (Int. List No. 162.1 and 163) in the year of 1965 are shown in Fig. 2. The differences between the incidence and mortality rate in the age groups under 70 years are considerable. The upper limit of operative measures is approximately 70 years. Table 1 shows the frequencies of resection and the 5 year survival rates in the age groups in the unselected series of lung cancer cases in two provinces of Sweden 1959–65. The result supports the assumption that the differences between the incidence and the mortality rate among patients in the age group under 70 years mainly depend on successful treatment. It is well known that epidermoid carcinoma has a more favourable prognosis than anaplastic carcinoma or adenocarcinoma. Table 2 shows however that in this series of primary lung cancer the distribution of histological types does not differ significantly between categories of patients under and over 70 years. The difference between the incidence and mortality in women is great even in the highest age groups, perhaps partly because the rates of morbidity

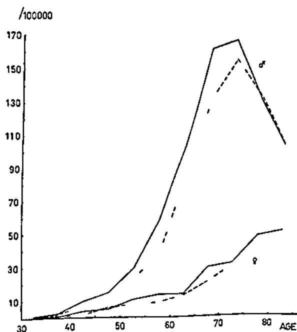


Fig. 2. Number of new cases of and deaths from lung cancer (Int. List Nos. 162.1 and 163) per 100 000 mean population in Sweden 1965 by age and sex.

——— incidence   – – – mortality

TABLE 1. *Frequency of Resection and 5 Year Survival Rate by Age. Unselected Cases of Primary Lung Cancer Diagnosed in Two Districts of Sweden, 1959–1965*

Age years	No. of cases	Resected (%)	5 year survival rate (%)*
0–49	93	38	14
50–59	203	30	10
60–69	399	26	7
70–	303	5	1
Total	998	21	6

* All 5 year survivors but one resected.

TABLE 2. *Distribution of Histological Types in Age Groups, Expressed as Percentages of Row Totals. Unselected Cases of Primary Lung Cancer Diagnosed in Two Districts of Sweden, 1959–1965*

Age years	Total no. of cases	Epidermoid carcinoma	Anaplastic carcinoma	Adeno-carcinoma	Other types
0–69	695	30	47	18	5
70–	303	30	50	16	4

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COMPARATIVE STUDIES OF THE CYTOLOGIC AND METABOLIC CHARACTERISTICS OF C3H MOUSE CELLS DURING "SPONTANEOUS" ALTERATION AND NEOPLASTIC CONVERSION IN VITRO

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During long term cultivation *in vitro* animal cells frequently undergo a number of morphological changes which are accompanied by the development of a capacity for continuous growth. This so-called phase of alteration is sometimes followed by a phase during which the altered cells apparently spontaneously develop tumorogenic properties. It is a matter of discussion whether the difference between these two phases of development is merely a difference of transplantability, or whether more fundamental changes of the altered cells are required before they become tumorogenic. In order to elucidate this problem the cytologic, metabolic, and growth characteristics of C3H mouse cells undergoing "spontaneous" alteration and neoplastic conversion *in vitro* were investigated. Quantitative studies of cell dimensions, number of nuclei and nucleoli, percentage of mitotic abnormalities and of chromosomal abnormalities revealed no characteristic differences between altered, non-tumorogenic cells and tumorogenic cells. The most striking cytogenic abnormality was the appearance of an unusually large number of biarmed chromosomes in one of the non-tumorogenic cell lines (C3H M), which showed an accrocentric mode at 36, a subtelocentric mode of 3 and a metacentric mode of 26. This abnormality persisted after the development of tumorogenic properties. Although the rate of glycolysis could not be correlated with the rate of tumorogenicity, when four different cell lines were compared, the development and increase of tumorogenic properties within two individual cell lines was accompanied by an increase in the glycolytic capacity. The glycolytic activity did not correlate with the rate of growth as measured by the doubling time under standard conditions of cultivation. However, if pCO_2 was raised to 10 per cent atm only cells with a high rate of glycolysis were able to maintain their normal rate of growth. It is suggested 1) that an increased glycolytic capacity may be necessary to maintain a high growth potential of the cells under *in vitro* conditions, where they might be exposed to low O_2 and high CO_2 tensions 2) and that a high growth potential may be essential for tumour production if, as in the case of the present experiments, immunological barriers have to be overcome when the malignancy of the cultured cells is tested in transplantation experiments.

Received 17 iv 71

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tality figures in statistical studies of primary lung cancer from both quantitative and qualitative points of view

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The author is obliged to Miss *Anne Marie Bolander, statistician,* Stockholm, and Dr *Einar Pedersen,* Oslo for expert criticism

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most of the bottom of the flasks the cells were transferred to new Fibiger flasks without plasma *For this and subsequent* transfers the cells were brought into suspension by gentle scraping without the use of trypsin When large amounts of cells were needed *the cultures were* transferred to Roux bottles in which up to 40 × 10[4] cells could be [illegible] 10 ml of [illegible] times weekly Except for the first passage the cells were grown directly on glass without plasma

Determination of Tumorogenicity

The tumorogenicity of the cultured cells was tested by their subcutaneous inoculation into the interscapular region *of new born (less than* 24 hours of age) inbred mice of the C3H strain On each occasion the medium was renewed within 24 hours of inoculation *The cells were* harvested by trypsinization and washed twice in Tyrode s solution Each mouse received 5 × 10[6] cells suspended in 50 microliters of Tyrode s solution.

The animals were examined at least *once* weekly All animals showing palpable tumours 2 weeks after inoculation were registered as takes Cases in *which palpable* tumours disappeared *before the* end of the second week after inoculation were considered negative since it was felt that no safe *distinction* could be made between *inflammatory* reactions and true tumour formation at this early stage without histological examination Animals with palpable tumours after 2 weeks which disappeared before the end of 3 months of observation were registered as takes with regressive tumour growth Animals which died before the second week were disregarded

Methods of Cytologic Investigations

Cellular dimensions were estimated by the determination of dry *weight protein content* and cell volume using 10 cultures in the exponential growth phase for each determination The dry weight of the *cells was measured at least* three times at *daily* intervals after drying at 100°C until constant weight was obtained Protein determination were *made as described by Oyama & Eagle* (26) whose method is based on that of Lowry using a phenol reagent (Folin Ciocalteau for colour development *The volume of* packed cells was measured in MSE microhaematocrit tubes after centrifugation *for* 15 minutes at 3500 × g in a MSE "Minor" centrifuge *with a special* micro-haematocrit head Cell counts were made in a Burker Turk *haemocytometer*

The nuclear characteristics were studied in living *cells* grown on 13 × 50 cm glass slides placed in Fibiger flasks Nuclear *volume was* measured under the phase contrast microscope (magnification *400 ×*) using a micrometer with units representing 1/5 of a micron under the *conditions of the present experiments* The longest and shortest axis of ten nuclei in each of ten cultures were measured, and assuming that the nuclei form a prolate spheroid the *volume* was calculated *according* to the formula $4/3 \pi \ (a/2) \ (b/2)^2$, where a and b are the longest and the shortest axis respectively

The number of nuclei and nucleoli *per cell was counted* under the phase contrast microscope (magnification 1000 ×) in one hundred cells from each of ten cultures Mitotic abnormalities were studied in *haematoxylin-eosin stained preparations.* One hundred mitotic figures were examined in each of ten cultures [illegible] after 4 hours of [illegible] with [illegible] *fixed in modified Carnoy s fluid, consisting of* glacial acetic acid and 99 per cent ethanol (1 3) and stained with Giemsa stain

Methods of Metabolic Studies

For metabolic studies the cells were harvested 24 hours after the last renewal of the medium in the same way as used in the inoculation tests for tumorogenicity *After washing the cells were resus-*pended in Ringer Locke s solution fortified with Tris (30 mM) Glycolysis was studied by measurements of glucose consumption and lactate accumulation in cell suspensions in Erlenmeyer flasks which were placed in a water bath shaker at 37°C Glucose and lactate concentrations were measured *every 30 min for 2–3 hours using specific enzy*matic methods as described by *Hugget & Nixon* (18) and *Horn & Bruns* (16) The reagents were *purchased from Boehringer* Mannheim Germany Cellular respiration was studied with conventional Warburg technique (37)

Growth Studies

The doubling time was determined by counts in Burker Turk s haemocytometer of ten trypsinized cultures selected at random on four consecutive *days The ability* of various metabolites to substitute for glucose was studied in the synthetic medium fortified with 20 per cent of foetal bovine *serum* which had been dialysed against running water for 2 hours and a balanced salt *solution for* [illegible] phate [illegible] d for [illegible] cell growth. The bicarbonate concentration was adjusted *to the CO_2 tension* to yield a pH of 7 4 Cultures in Fibiger flasks *loosely stoppered* with cotton were placed in large glass cylinders *ten in each The cylinders* were closed, placed in the incubator and *perfused continuously* with gas

The apparently spontaneous neoplastic conversion of mammalian cells propagated *in vitro* was first reported by *Gey* (12, 13, 14) and *Earle* (7, 8) Similar observations have later been published by a number of authors (for review, see *Sanford* 31, 32) The cause of this "spontaneous" neoplastic conversion is not known, and in spite of all efforts it has not been possible to correlate the development of tumorogenic properties with any distinct cytologic, metabolic or immunologic change

Cultures of animal cells frequently pass through five phases of development 1) The initial outgrowth of a mixed cell population 2) The sluggish growth phase during which many cultures perish 3) The development of a more homogeneous cell culture with increased growth activity 4) The phase of *alteration* characterized by cytologic and structural changes and by a further acceleration of growth rate 5) The development of tumorogenic properties

According to *Hayflick* (15) *altered* cell lines with a capacity for continuous growth *in vitro* should be considered malignant, even if transplantation experiments fail to reveal their tumorogenicity *Barski* (3), on the other hand, has stressed that alteration and malignant conversion are not equivalent The same opinion is held by *Sanford* (31, 32) At the present moment this discussion remains rather academic, because our criteria of malignancy *in vitro* are still not satisfactory (for review, see 19, 33) The only proof of neoplastic conversion is the demonstration of the tumorogenic properties of cultured cells inoculated into susceptible hosts, but negative results of transplantation experiments of this kind are not conclusive

Numerous factors such as histocompatibility, host provided nutritional conditions vascular reactions, and others may play a decisive role in the outcome of the transplantation test More recently it has been shown that long term infection of cultured tumour cells with mycoplasma (10) and Rauscher leukaemia virus (1) may reduce their tumour producing capacity

In our laboratory numerous cell lines have been studied in order to clarify whether tumorogenicity is the only characteristic difference between cells in the fourth and the fifth phase of development mentioned above In the present work the cytology, energy metabolism and growth of four permanent C3H mouse cell lines were investigated at different stages of their neoplastic conversion Antigenic changes have been described in a previous report (21) and will be discussed in greater detail in later communications

MATERIAL AND METHODS

Material

Primary explants were prepared by careful scissoring of mixed tissues from a litter of 19 day-old C3H mouse embryos, and of the lung and spleen from a normal adult C3H mouse From these cultures three cell lines were established i e the C3H E cells derived from the embryonic tissues the C3H M cells from the spleen and the C3H Li cells from the lung For comparison Earle s strain L 929 fibroblasts were included in this investigation These cells were obtained from Dr *Earle s* laboratory in 1957 They were derived from the subcutis of a normal C3H mouse After treatment for 111 days in 1940–41 with methylcholanthrene they were found to be tumorogenic in C3H mice (8)

Methods of Cultivation

The explants were embedded in chicken plasma clots in Fibiger flasks which are a 75 ml modification of the Carrel flask with a bottom area of 25 cm² In each flask 20 30 pieces of tissue measuring about 1×1 mm were placed After coagulation each culture received 5 ml of a semisynthetic medium (Fib 14 B) containing 20 per cent foetal bovine serum and 80 per cent of Eagle s minimum essential medium (6) with a two-fold concentration of the essential amino acids and a four fold concentration of the vitamins and of glutamine All chemicals for the synthetic medium were purchased from Sigma St Louis Mo U S A and the serum from Flow s Laboratories, Glasgow Scotland Penicillin and streptomycin generously supplied by Lovens Kemiske Fabrik Copenhagen Denmark, were added at concentrations of 150 000 i u /l and 25 mg/l respectively The cultures were gassed with a mixture containing 5 per cent CO_2 20 per cent O_2, and 75 per cent N_2 purchased from Dansk Ilt & Brintfabrik Copenhagen After the development of a monolayer of cells covering

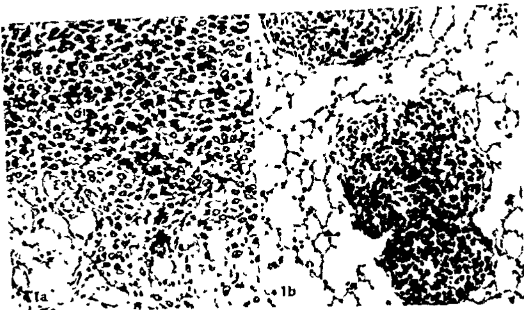


Fig 1 Tumour (a) and pulmonary metastases (b) produced in a newborn C3H mouse inoculated subcutaneously with 5 × 10⁴ L-929 cells propagated *in vitro* for 25 years Haematoxylin-eosin staining Magnification 300 × (a) and 200 × (b)

The L 929 cells which were studied for comparison, caused progressively growing tumours in 47 per cent of newborn mice (Table 1) Histologically the tumours resembled those produced by the other three cell lines and pulmonary metastases were occasionally seen (Figs 1 a b)

Cytologic Characteristics of Cells in vitro

All four cell lines were typical fibroblast like cultures which at no time during the experimental period could be distinguished from one another by routine microscopy However quantitative studies revealed various differences

The dimensions of the cells as measured by dry weight protein content, and cell volume are presented in Table 2 The dry weight and protein content of the C3H L1 cells which were the most tumorogenic were significantly larger than those of the three other cell lines but the cell volume did not differ significantly However, the development of increasing tumorogenicity in the course of the experimental period was not accompanied by any significant increase in the dimensions of the C3H-E and C3H M cells The Table also shows differences in nuclear volume after 6 months of cultivation, reflecting the change in chromosome number to be described below (Table 3) After 36 months of cultivation these differences had deminished or disappeared The percentage of multinuclear cells did not exceed 3 in any of the cell lines No significant differences between the non-tumorogenic C3H-M and the tumorogenic C3H L1 cultures were observed in this respect The mean number of nucleoli per cell varied from 3 5–4 5 It was a little higher in the C3H E cells than in the C3H M and L 929 cells, but the differences were small The percentage of mitotic abnormalities varied from 1 6 per cent to 4 5 per cent Various types of spindle failure and chromosomal bridge formation were the dominant abnormalities which were accompanied by a considerable degree of aneuploidy in all four cell lines (Table 3) However, in most cases a modal value could be established

mixtures containing 20 per cent O_2 and 0–10 per cent CO_2

RESULTS

The primary explants of mixed embryonic tissues, and lung and spleen tissue from the same adult C3H mouse were cultured in plasma clots and semisynthetic fluid Fib 14 B medium as described Rapid growth of a mixed cell population was seen soon after explantation, and it continued until the clot areas were covered with a dense monolayer of cells

After this initial growth phase the cells were transferred without trypsinization to new culture vessels in which they adhered directly to glass The transfer was followed by the usual second phase of sluggish growth during which a number of cultures died In the surviving cultures a more homogeneous fibroblast like population eventually developed and after a few passages three permanent cell lines (C3H-E, C3H-M, and C3H-L1), which could be devided once weekly, were established Three to four months after explantation, cellular islands were observed in which a shortening of the cell processes and the development of more polygonal shapes together with an increase in refractive index were characteristic findings At the same time it became easier to bring the cells into monodispersed suspensions without clumping The cultures were soon overgrown by these altered cells and could now be devided 2–3 times weekly

Tumorogenicity of Cultured Cells in Mice of the Original Donor Strain

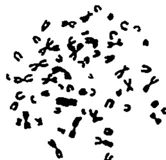
The tumorogenicity of the four cell lines under investigation in newborn C3H mice is summarized in Table 1 After 6–8 months of cultivation, when all cell lines had undergone the alterations described above, progressively growing sarcomas were seen in 14 per cent and 100 per cent of newborn C3H mice inoculated with 5×10^6 C3H-E and C3H L1 cells, respectively However, the C3H M cells which were derived from the same donor as the C3H-L1 cells, did not produce progressively growing tumours at this time, but regressively growing tumours were seen in 20 per cent of the recipients

After 12–16 months of cultivation all three cell lines produced sarcomas in virtually 100 per cent of newborn mice inoculated Microscopic examinations frequently showed moderate signs of invasiveness and pulmonary metastases

TABLE 1 *Tumorogenicity* of Four Cultured C3H Mouse Cell Lines in Newborn C3H Mice*

Cell line	Culture age (months)	Number of mice without and with tumours 0	(+)	+	total
C3H E	6–8	19	17	6	42
	12–16	0	0	7	7
	24–48	11	1	37	49
C3H M	6 8	20	5	0	25
	12–16	0	0	32	32
	24–48	5	4	58	67
C3H L1	6–8	0	0	25	25
	12–16	1	0	45	46
	24–48	0	0	25	25
L 929	25 years	15	19	30	64

* Inoculum dosis 5×10^6 cells 0 = No palpable tumour 2 weeks after inoculation (+) = Regressive tumour growth after 2nd week + = Progressive tumour growth until death



2a



2b

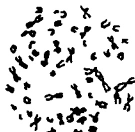


Fig 2 Karyotype from parental C3H-M line (a) and from 2 of its clones (b, c), all showing a near triploid chromosome number and a high percentage of metacentric chromosomes

TABLE 2 *Cytological Characteristics* of Four C3H Mouse Cell Lines Propagated in vitro*

Cell line	Culture age	Dry weight mg × 10⁻⁶/cell	Protein content mg × 10⁻⁶/cell	Cell volume μl × 10⁻⁶/cell	Nuclear volume μl × 10⁻⁶/nucleus	Nucl vol × 100 / Cell vol.
C3H E	6–8 mths	0 84±0 19	0 59±0 10	5 80±0 71	0 57±0 15	9 8
	36 mths	0 60±0 05	0 50±0 06	5 76±0 21	0 72±0 06	12 5
C3H M	6 8 mths	0 79±0 10	0 49±0 04	5 71±0 65	0 83±0 12	14 5
	36 mths	0 69±0 06	0 54±0 08	5 14±0 11	0 86±0 08	16 7
C3H L1	6–8 mths	1 23±0 23	0 91±0 12	5 45±0 68	1 07±0 21	19 6
	36 mths	0 91±0 12	0 77±0 11	4 86±0 11	0 89±0 07	18 3
L 929	25 years	0 53±0 09	0 47±0 06	4 25±0 68	1 03±0 64	24 2

* Mean values of ten cultures with the 95 per cent confidence interval

as shown in the Table A near diploid mode was only found in 6 months old C3H-E cultures

The chromosomes of the L-929 cells were all acrocentric The same was the case of the C3H E and C3H-L1 cells 6 months after explantation However, during later investigations of these two cell lines 1–5 biarmed chromosomes, mostly metacentric, were found in most cells The C3H-M cells, on the other hand, showed a considerable number of biarmed chromosomes at all times of investigation (Figs 2 a, c) In Table 4 the mode of acrocentric and biarmed chromosomes in the C3H M parental line and in five clones is presented

Metabolism

The results of the metabolic studies are presented in Table 5 As it is seen, the oxygen uptake was significantly higher in the L 929 and C3H-M line than in the two other cell lines The C3H L1 cells showed an increasing rate of respiration during the three year period of cultivation while the oxygen consumption of the C3H-E and C3H M cells remained constant

The glucose consumption, on the other hand, was significantly lower in the L 929 cells than in the C3H E and C3H L1 cells Also in the C3H-M cells a fairly low rate of glucose consumption was found 6 8 months after explantation, but the difference in

TABLE 3 *Changes in Chromosome Number in Four C3H Mouse Cell Lines during Propagation in vitro*

Cell line	Culture age (months)	No of metaphases counted	Spread of chromosomes	Median no of chromosomes	Mode
C3H E	6	101	33–101	41	41
	12	200	9 153	56	58
	36	80	77 109	94	96
C3H M	6	80	20 124	58	63
	12	258	31 86	56	60
	36	47	40 68	60	61
C3H L1	6	50	45–172	104	
	12	90	48 155	70	62
	36	105	42–107	100	99 102
L 929	25 years		28–180	64	64

TABLE 6 *Doubli ig Time* of Four C3H Mouse Cell Lines Propagated in vitro*

Culture age	*C3H E*	Doubling time (hours) C3H M	C3H L_1	L-929
6–8 mths	30 85+3 45	29 40+2 30	24 25±2 76	—
12 16 mths	26 50±2 58	29 30±3 60	22 00±3 57	—
36 mths	23 70±3 60	29 80±3 30	22 70±1 30	—
25 years	—	—	—	29 15±3 22

* Median values of ten experiments and 95 per cent confidence interval

and respiration for the growth of C3H E and C3H L1 cells might be different from that for C3H M and L-929 cells on the other hand

In order to elucidate this possibility two series of experiments were carried out In the first one the ability of lactate, lactate + oxalacetate and succinate to substitute for glucose in a semisynthetic medium fortified with 20 per cent dialysed foetal bovine serum was investigated The results are shown in Table 7 from which it is seen that glucose was essential for the growth of the two cell lines with the highest Warburg quotient *i e* C3H E and C3H L1 On the other hand in 6 8 month old C3H M cultures and in L 929 cultures lactate + oxalacetate could substitute for glucose to a great extent while lactate alone was less efficient and succinate was without any growth promoting effect After *the development of tumorogenicity* and a higher Warburg quotient the growth of the C3H M cells also became dependent on the presence of glucose in the medium

In the second series of experiments the influence of pCO_2 on the growth of the four cell lines was investigated From Table 8 it appears that CO_2 was essential for the growth of all four cell lines The optimal CO_2 tension was 5 per cent atm At 10 per cent CO_2 a cytostatic effect was seen in cultures with a low Warburg quotient *i e* 6–8 month-old C3H M cultures and the L-929 cultures while no significant growth inhibition was seen *in the* C3H E and C3H L1 cultures After the shift of the metabolic balance towards a higher rate of glycolysis in the C3H M cells, these also became resistant to the cytostatic effect of high CO_2 tensions

DISCUSSION

Searching for criteria of malignancy in cultured *cell lines it should be borne in mind* that the development of tumorogenic properties may depend on a large number of alterations leading to different degrees of malignancy The present quantitative cytologic studies failed to reveal any characteristic differences between altered but non tumorogenic cells and tumorogenic cells However, this does not necessarily exclude the possibility that the morphological changes represent an essential part of the process of malignization and that cells in the fourth phase of alteration are indeed malignant The development of new antigenic properties (21) may explain their failure to produce progressive tumour growth in animals of the original donor strain but the present results indicate that the development of a higher glycolytic capacity may enable the cells to overcome the immunological barrier

Cytology

The described cytologic and structural changes in the C3H E C3H M, and C3H L1 *cells* entering into the phase of alteration agree with those originally observed by *Gey (12 13 14) and Earle (7)* in rat and mouse cell cultures undergoing spontaneous alteration and by later authors studying neoplastic

TABLE 4 *The Modal Number of Acrocentric and Biarmed Chromosomes in the Parental Line and in Five Clones of the C3H M Mouse Cells after Three Years of Cultivation*

Cell line	Number of mitotic figures counted	Total		Number of chromosomes		
		Median	Mode	Acrocentric Mode	Subtelocentric Mode	Metacentric Mode
C3H M parental line	92	60	61	36 (22–38)	3 (2–7)	26 (21 30)
Clone 2	100	57	55	34 (24–44)	4 (3–7)	22 (16 27)
Clone 5	100	56	58	35 36 (23–41)	4 (2 6)	22 (17 42)
Clone 8	*113*	57	57	35 (22–41)	4 (2 8)	26 (16 28)
Clone 12	104	58	58	33 (26–47)	4 (2–7)	22 (16 27)
Clone 15	107	56	56	36 (22–42)	4 (1–6)	22 (15 25)

The figures in *parentheses indicate the spread*

relation to the C3H-E and C3H L1 cells was *not statistically significant* After three years, the C3H-E and C3H-M cells showed a significant increase in glucose consumption

The lactate production was significantly lower in the L 929 fibroblast and in the C3H-M cells 6–8 months after explantation than in the two other cell lines After three years of cultivation a significant increase was seen in the C3H-M cells

The Warburg quotient, *i e* the ratio of lactate production to oxygen consumption, was significantly higher in the C3H-E and C3H-L1 cultures than in the L 929 and the C3H-M cultures However, after three years of cultivation a doubling of this quotient was seen in the latter cell line

Growth Rate

The growth rates of the four cell populations were compared by measurements of their doubling time From Table 6 it is seen that the doubling time of 6–8 month old C3H-E and C3H-M cultures was a little longer than that of the C3H L1 cultures Later on, an acceleration of the growth of the C3H E cells occurred while that of the C3H-M cells remained unchanged After 1–3 years of cultivation the C3H E and C3H L1 cells showed a doubling time which was about 25 per cent shorter than that of the C3H M and L 929 cells

The differences in the Warburg quotient indicated that the importance of glycolysis

TABLE 5 *Metabolism of C3H Mouse Cells Propagated in vitro*

Cell line	Culture age	Oxygen consumption	Glucose consumption	Lactate production	*Lactate* Oxygen
		(micromoles per mg dry weight per hour)*			
C3H E (embryonic)	6 8 mths	0 19±0 04	0 43±0 08	0 83±0 19	3 97±0 94
	3 years	0 23±0 07	0 68±0 07	1 08±0 11	3 96±1 10
C3H M (spleen)	6 8 mths	0 43±0 10	0 32±0 05	0 41±0 09	0 87±0 20
	12 16 mths	0 45±0 06	0 43±0 09	0 62±0 21	1 50±0 73
	3 years	0 43±0 04	0 51±0 08	0 69±0 12	1 77±0 42
C3H L1 (lung)	6 8 mths	0 22±0 03	0 42±0 09	0 67±0 13	3 39±0 71
	3 years	0 32±0 04	0 48±0 06	0 87±0 14	*2 85±0 91*
L 929	25 years	0 51±0 06	0 23±0 08	0 32±0 13	0 67±0 39

* Median values of ten experiments and 95 per cent confidence interval

TABLE 8 *The Influence of pCO_2 on the Growth of Cultured C3H Mouse Cells with Different Warburg Quotients*

Cell line	Culture age	Warburg's quotient $(\frac{I}{O})$*	Initial cell no × 10 per culture*	Final cell no × 10 and growth index $(\frac{C}{C_i})$			
				0 % CO_2	1 % CO_2	5 % CO	10 % CO_2
L 929	25 years	0.67±0.39	1.85±0.29	1.70±0.36 (0.92)	3.35±0.78 (1.81)	5.32±0.48 (2.88)	2.96±0.51 (1.60)
C3H M	6 8 mths	0.87±0.20	0.49±0.08	0.13±0.03 (0.27)	0.90±0.27 (1.84)	1.95±0.45 (3.98)	0.94±0.21 (1.92)
C3H M	3 years	1.77±0.42	0.44±0.11	0.11±0.02 (0.25)	1.07±0.05 (4.70)	2.70±0.25 (6.14)	2.46±0.39 (5.59)
C3H L1	6 8 mths	3.39±0.71	0.48±0.18	0.73±0.20 (1.50)	1.61±0.52 (3.4)	1.89±0.44 (3.9)	1.81±0.66 (3.8)
C3H E	6 8 mths	3.97±0.94	1.82±0.32	1.58±0.38 (0.87)	4.76±0.74 (2.62)	6.20±1.41 (3.41)	5.84±1.25 (3.21)

* Median values of ten cultures and 95 per cent confidence interval

TABLE 7 *Warburg Quotient and Growth Dependency on Glucose of Four Lines Propagated in vitro*

Cell line	Culture age	Warburg's quotient $(\frac{L}{O})$*	Initial cell conc (cell no × 10^6 per culture)*	Cell conc after 3–5 days of incub (cell no × 10^6/culture)*				
				Substrates substituting for glucose				Control
				0	Lactate (5 mM)	Succinate (5 mM)	Lactate (5 mM) + oxalacetate (1 mM)	Glucose (5 mM)
L 929	25 years	0 67±0 39	2 33±0 28	0 92+0 29	2 30±0 25	1 43±0 77	4 19±0 79	4 56±0 63
			1 60±0 29	1 63±0 30	2 12±0 25	1 38±0 24	2 55±0 45	3 92+0 38
C3H M	6–8 mths	0 87±0 20	1 02±0 15	0 67±0 18	1 90±0 27	0 95±0 30	2 40±0 25	2 90±0 30
C3H I 1	6–8 mths	3 39±0 71	1 06±0 15	0 20±0 09	0 81±0 02	0 28±0 06	0 84±0 10	2 24+0 67
C3H F	6–8 mths	3 97+0 94	2 48±0 41	0 08±0 03	0 09±0 03	0 08±0 05	0 14±0 07	8 76±2 46

* Median values of ten cultures and 95 per cent confidence interval

The present results seem to agree with this conclusion Mitotic and chromosomal abnormalities occurred in all cultures tumorogenic as well as non tumorogenic in spite of the facts that trypsin was never used for propagation of the cells and that foetal calf serum was used throughout

One year after the termination of the present experimental period the C3H L1 cells showed a shift from a near pentaploid mode towards a near triploid mode This coincided with an accidental mycoplasma contamination which may have had an influence on the chromosome number of the cultured cells Thus *Fogh & Fogh* (11) found a reduction in the chromosomal number of human cells from 70–76 to 63–68 as a consequence of mycoplasma infection At later investigations a further reduction of the chromosomal mode of the C3H L1 cells to near diploid values has been observed In spite of this reduction in chromosome number the cell strain is still tumorogenic

The most striking chromosomal abnormality in the present study was the presence of a large number of biarmed chromosomes in the C3H M line both before and after the development of tumorogenicity Biarmed chromosomes also appeared in the tumorogenic C3H E and C3H L1 line but at a later date and in a much smaller number as reported in other cell lines by other authors (3 9 22 25 27) The majority of the biarmed chromosomes were metacentric suggestive of isochromosome formation This agrees with previous findings (17 27) The mechanism through which the biarmed chromosomes are formed is unknown However *Hsu et al* (17) have proposed that breakage in the centromeric region of a chromosome followed by fusion of the broken ends of sister chromatids may explain the appearance of metacentrics in C3H bone marrow *in vitro* *Parshad & Sanford* (27) have found that the frequency of minutes was higher than that of metacentric chromosomes indicating that most metacentric chromosomes originated through two breaks

Metabolism and Growth

In primary explants of non malignant tissues a temporary decrease in oxygen consumption and a considerable increase in glycolytic activity has been observed by a number of authors (see 5, 29, 30, 38, 39) Permanent cell lines often show a relatively high rate of glycolysis and a distinction between non malignant and malignant cell cultures on the basis of metabolic studies is usually not possible However neoplastic conversion of individual cell lines in *vitro* and the development of increasing tumorogenic properties have been reported to be accompanied by a marked increase in glycolytic capacity (33 41) In the present investigation the comparison of four different cell lines did not reveal any correlation between metabolism and tumorogenicity Two groups of cells could be distinguished the non tumorogenic 6 8 month-old C3H M cultures and the tumorogenic L-929 cultures which showed a higher rate of respiration and a lower rate of glucose consumption and/or lactate production than the slightly tumorogenic 6 8 month-old C3H E cultures and the highly tumorogenic C3H L1 cultures Consequently the metabolic balance as expressed by the Warburg quotient was 4 5 times higher in the latter group than in the former

However the development of tumorogenic properties in the C3H M cultures was accompanied by a significant increase in glycolysis and Warburg quotient and in the C3H E cultures the rate of glucose consumption rose by 58 per cent at the same time as the percentage of inoculated newborn mice producing progressively growing tumours rose from 14 to 75 per cent These observations indicate that for growth *in vivo* as tumours, an increase in the glycolytic capacity of the cultured cells may be required as suggested by previous authors (33 41)

It is conceivable that increased glycolytic capacity provides the cells with a high growth potential enabling them to overcome immunological and other environmental barriers *in vivo* As described in a previous report (21)

conversion in vitro (for review, see 31) However, these changes were not necessarily accompanied by the development of tumorogenic properties At least this was not the case with the C3H-M line

Lewis (23) has summarized the cytologic characteristics of tumour cells *in vitro*, stressing the increased cellular, nuclear, and nucleolar dimensions and the frequent occurrence of multinucleated cells and mitotic abnormalities *Barker* & *Sanford* have recently reported (2) that cells undergoing malignant conversion *in vitro* showed a progression of a number of cytologic changes including increases of the number and size of nucleoli and of the nuclear cytoplasmic ratio The tumorogenicity was demonstrated by intramuscular implantation into whole body irradiated adult animals or by implantation into the anterior eye chamber of unconditioned adult recipients

The quantitative study of cell size nuclear size, number of nuclei and of nucleoli in the present investigation failed to demonstrate any clear correlation between cytologic changes and tumorogenicity Various explanations may be offered for this apparent discrepancy First of all it should be stressed that many of the cytologic characteristics reported by others were noticed in our material when the cells passed from the third phase into the fourth phase of alteration mentioned above We only failed to demonstrate any progression of the cytologic changes when the altered cells became tumorogenic Differences in the classification of the material as malignant may also play a role As will be discussed in greater detail in a later communication the antigenicity of the implanted cells the age and the conditioning of the recipient and the site of implantation are factors of great importance for the demonstration of the tumorogenic properties of cultured cells Finally it may be pointed out that cytologic alteration of a minor fraction of a heterogeneous cell population may not be revealed in a quantitative study of non selected cells Thus for diagnostic purposes a qualitative cytologic analysis seems more suitable but for the comparison with results of metabolic investigations quantitative data are necessary

The number of abnormal mitotic figures of 6–8 month old cultures did not exceed that observed by *Levan* & *Biesele* (22) in newly set up cultures of embryonic murine tissues and no significant difference between the non tumorogenic C3H M cultures and tumorogenic C3H L1 cultures was observed

Chromosomal changes frequently occur in cultured cells malignant as well as non malignant *Levan* & *Biesele* (22) concluded from their detailed studies of murine cells *in vitro* that even with the mildest conditions of preparation newly set up tissue cultures will exhibit a considerable frequency of mitotic disturbances indicating the existence of an unavoidable background mutation rate which may be increased by changing the environment

Some of the environmental factors which have been reported to influence karyotypic stability may also have an effect on neoplastic conversion Thus a number of observations suggest that trypsinization may enhance chromosomal changes as well as malignant conversion (3 22) On the other hand foetal calf serum as compared to other serum supplements in the medium seem to delay the development of both chromosomal deviations and tumorogenic properties (9 25 27) It has also been shown (9 27) that an increase in the frequency of abnormal chromosomes sometimes occurs just before or immediately after the onset of neoplastic conversion

Although these observations tend to suggest a correlation between the development of tumorogenicity and chromosomal aberrations it remains a fact that chromosomal alteration may occur long before tumorogenic properties can be demonstrated by the methods used so far It is therefore assumed by most authors (9 25 27 28 31 32) that no general correlation exists between neoplastic conversion *in vitro* and numerical and structural chromosomal abnormalities

to overcome the reported immunological barrier (21) and kill the host. The relatively high tumorogenicity shown by the L 929 cells in spite of their low Warburg quotient and their sensitivity to high CO_2 tensions does not contradict this tentative conclusion because this very old cell line seems to be less immunogenic in C3H mice than the remaining three cell lines. The immunological barriers and thus the need for an increased growth potential seemed to be smaller in the case of L-929 cells.

The primary purpose of the present study was to elucidate the question whether alteration is equivalent to neoplastic conversion. As described, we were unable to demonstrate any quantitative cytologic changes associated with the development of tumorogenic properties in cultures passing from the phase of alteration into the final phase of tumorogenicity. However, an increased glycolytic activity seemed to provide the altered cells with an increased growth potential *in vivo*, which enabled them to overcome immunological barriers and produce progressively growing tumours. This represents a difference in transplantability, but not necessarily in malignancy. The development of more sensitive tests for tumorogenicity might reveal that alteration after all is equivalent to neoplastic conversion.

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the present cell lines proved to be immunogenic in the original strain of C3H mice Thus the manifestation of tumorogenicity might depend on the rate at which the inoculated cells multiply before the immune response of the host reaches its maximal level

In the present investigation alteration *in vitro* was accompanied by the well known gradual acceleration of cell growth However, 6–8 months after explantation only *small differences could be demonstrated* between the doubling times of the non tumorogenic C3H M, the slightly tumorogenic C3H E and the highly tumorogenic C3H L1 cells This is in agreement with our previous studies of their replication time determined by the colcemid method (34) and their generation time studied by autoradiography (35)

The development of a higher degree of tumorogenicity and glycolytic activity was accompanied by a shortening of the doubling time of the C3H E cells but not of that of the C3H M cells under standard conditions of cultivation Thus these observations do not *give much support to the hypothesis that the* growth potential was a limiting factor in tumour production

However, *in vivo* and *in vitro* conditions are not directly comparable Low oxygen tensions due to insufficient vascularization and high CO tensions may be growth inhibiting factors playing a role *in vivo* but not under the present *in vitro* standard conditions If this is the case a high glycolytic capacity may become essential for progressive tumour growth

Even under optimal *in vitro* conditions the necessity of glycolytic energy production for the growth of cells with a high Warburg *quotient* was indicated by the fact that substrates for respiratory energy production were unable to substitute for glucose as growth factors *in vitro In vivo,* an insufficient oxygen *supply would* make glycolysis an even more important source of energy and so would the cytostatic effect of high CO tensions which have been proposed by *Loomis* (24) to function as a physiological regulator of cell growth, to which malignant cells have become resistant

The present results *seem to confirm* Loomis theory with the modification that resistance to the cytostatic effect of high CO_2 tensions is characteristic of cells with a high *glycolytic activity* which include most but not all tumour cells Thus the growth of the C3H E and C3H L1 cells was not significantly inhibited by 10 per cent CO and similar observations have previously been made with cultures of Ehrlich s ascites tumour (4) All of these cell lines had a high Warburg quotient Also the C3H M cells became resistant to suppression by 10 per cent CO when their glycolytic capacity increased However the L 929 cells which were tumour producing but had a low Warburg quotient remained sensitive to the cytostatic effect of high CO tensions

The cytostatic activity of high CO tensions might be explained by their strong antirespiratory effect previously reported from this laboratory (for reference see 20) Glycolysis on the other hand is either stimulated *or remains uninfluenced* by high CO_2/HCO_3 concentrations (20 36 40) Under such circumstances glycolysis may become a more important source of energy than respiration

No measurements of pCO_2 *and* pO_2 *were* made in tumours produced by the present cell lines However it seems reasonable to assume that the tumours produced by 6 8 month old C3H E cultures were *suffering* from an insufficient oxygen supply These tumours grew very quickly but most of them regressed in 2–4 weeks with ulceration and suppuration of completely necrotic *tumour* material However the small tumours seen in 20 per cent of newborn mice inoculated with 6 8 month old C3H M cultures grew very slowly and soon regressed without perforation of the skin It seems more likely that a high pCO rather than a low pO was the growth limiting factor in these tumours But in both cases the *development* of a higher glycolytic capacity was accompanied by an increased percentage of progressively growing tumours which were able

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STUDIES OF THE GOITROGENIC AND ONCOGENIC EFFECT OF METHYLTHIOURACIL IN C3H MICE

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Methylthiouracil (4 Methyl 2 thiouracil MTU) had a strong goitrogenic effect in C3H mice Metabolic studies showed a significantly higher consumption of oxygen in thyroid gland tissue after treatment with MTU Malignant changes were not observed The serum of MTU and Thycapzol treated mice showed a higher capacity to bind T3—indicating a lower production of endogenous T4

In a previous publication (*Jemec* 1970) it was reported that Thycapzol (1-methyl-2-mercapto-imidazol) had a moderate goitrogenic effect in C3H mice However, neither histological nor metabolic studies revealed any signs of direct or indirect carcinogenic effect on the thyroid gland

In the present investigation similar studies were carried out with Methylthiouracil (MTU 4 methyl 6-oxo-2 thio 1, 2, 3, 6-tetrahydrogenpyrimidine) In addition the effect of Methylthiouracil and Thycapzol on thyroid hormone production was compared

MATERIALS AND METHODS

A total of 319 inbred C3H/FIB mice were divided into three groups

Group I 176 mice (82 ♀ and 94 ♂) received MTU in their drinking water as a 0 1 per cent solution from the age of two months Fresh solutions were prepared once weekly, and no other fluid was supplied

Methylthiouracilum (Pd D WHO, BP, USP, NFN Inn) is a white crystaline powder of bitter taste practically insoluble in water at neutral pH Therefore pH of the demineralized drinking water was adjusted to pH 7 8 by a phosphate buffer (K_2HPO_4-2 28 g and KH_2PO_4-1 36 g per litre) In this solution saturation with MTU was obtained at a concentration of 0 1 per cent

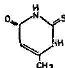


Fig 1 4-methyl-6-oxo-2-thio 1, 2, 3, 6-tetrahydrogenpyrimidine (Methylthiouracil)

Group II 6 C3H mice received Thycapzol in their drinking water for 2 years. Thycapzol was dissolved in demineralized drinking water without buffer at a concentration of 0 05 per cent

Group III 137 strain C3H mice (53 ♀ and 84 ♂) served as a control group which did not receive any drugs

All animals received ad libitum rat pellets – Rostock mixture purchased from Korn og Foderstof Kompagniet, Copenhagen Analyses made in the National Food Institute in Copenhagen showed that these pellets contained 9–10 mcg of iodine per gramme This figure, incidentally, represents 50 to 100 times the iodine content in rat fodder recommended by the National Academy of Science

Beginning during the 5th month of treatment, groups of 7–12 mice were selected at random at

Received 5 iii 71

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TABLE 1 *Pathological and Histological Findings*

	Total 313			
	treated		non treated	
	82	94	53	84
	♀	♂	♀	♂
	176		137	
Thyroid hyperplasia	82	94		
Thyroid adenoma	1	1		
Unilat aplas of thyr gl		2	1	1
Cystis renis	3	2	1	2
Hepatoma	4	2	2	1
Cystis ovarii	11		5	
Tumour of the mammae	8		3	

causes They all showed considerable hyperplasia of the thyroid gland like all the other treated animals

The influence of MTU treatment on the weight of the thyroid gland is shown in Fig 2 As seen, a significant increase was observed already after 5 months of treatment—reaching a maximum after 10 months (Fig 3) Apart from two adenomas after 16 months of treatment with MTU, no thyroid tumours were found

Microscopic changes occurred within 8 months The low cuboidal epithelium developed into columnar epithelium, and the colloid changed from a solid into a vacuolated and spongy substance The acini themselves looked shrivelled, the colloid having shrunk considerably

The Adenoma

The adenomas resembled those described by *Morris et al* (1951), the acini being smaller and separated by a structureless stroma The cells were smaller, and the nuclei were hyperchromatic No pathological mitotic figures were seen, and no invasive growth was observed (Fig 4)

Metabolic Studies

The tissue of the thyroid glands of mice treated with MTU showed a significant in



Fig 3 A Thyroid gland of a non treated C3H mouse B Thyroid gland of a C3H mouse treated for 12 months with Methylthiouracil.

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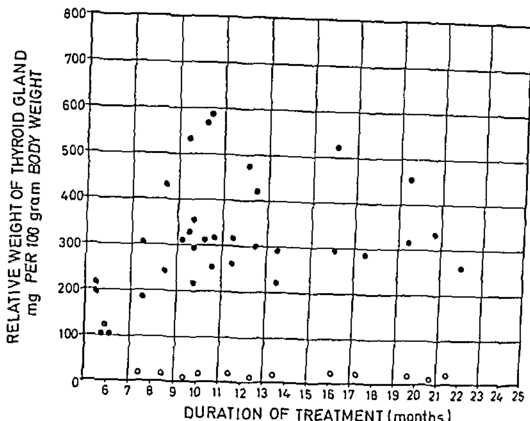


Fig 2 ● Methylthiouracil treated mice (Each point represents one thyroid gland) ○ Non treated controls (Each circle represents an average of 6–10 thyroid glands) The influence of Methylthiouracil treatment on the weight of the thyroid gland in C3H mice The weights of the thyroid glands in the two groups were significantly different (< 0.01) No significant differences in the total body weights of the treated and non treated animals were observed

various time intervals After killing the animals were weighed and with the use of a magnifying glass the thyroid gland of each animal was removed and weighed Microscopic examination was made of the thyroid glands the lungs and any tissue showing gross pathological changes

Metabolic studies The respiration of the thyroid gland of one treated mouse in comparison with that of 6 to 10 non treated thyroid glands of control mice was measured using conventional Warburg technique Glucose consumption and lactate production were studied simultaneously with the specific enzymatic methods described by *Hugget & Nixon* (1956) and *Horn & Bruns* (1956) All results were calculated per mg dry weight (d w) Reagents were purchased from C F Boehringer & Soehne GMBH, Mannheim Germany Endogenous thyroid hormone (T_4) saturation of the blood of Thycapzol treated and MTU treated mice was compared with non treated control mice by means of the Thyopac–3* test described by *Cavalieri et al* (1969) Kits for T_3 uptake testing were purchased from the Radio chemical Centre Amersham U K

Statistical significance (P) was evaluated on the basis of Wilcoxon s rank sum test 95 per cent confidence intervals ($\pm$ 2 e) were calculated according to *Dean & Dixon* (1959)

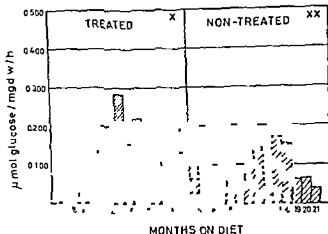
RESULTS

Pathological and Histological Findings

Table 1 summarizes the pathological findings made at autopsy of MTU treated and non treated mice As seen, no malignant tumours were found in the thyroid glands in either of the two groups The MTU treated mice showed a higher number of hepatomas, tumours of the mammae and ovarian cysts, but the difference between treated and non treated mice was not statistically significant A large number of treated mice (19 ♀ and 24 ♂) died spontaneously from unknown

* trade mark Code IM 62

Fig 6 The influence of Methyl thiouracil treatment on glucose consumption of thyroid gland tissue × Each column represents one thyroid gland ×× Each column represents an average of 6–10 thyroid glands



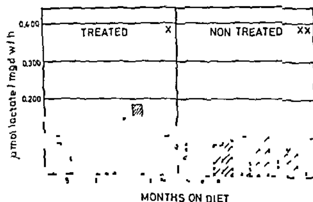


Fig 7 The influence of Methylthiouracil treatment on lactate production in thyroid gland tissue. × Each column represents one thyroid gland ×× Each column represents an average of 6–10 thyroid glands

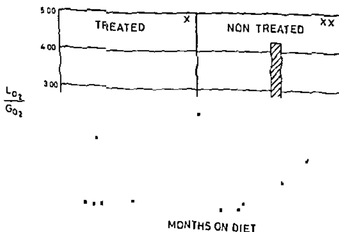


Fig 8 The influence of Methylthiouracil treatment on the ratio of lactate production to glucose cousumption ($\frac{L_{O_2}}{G_{O_2}}$) in thyroid gland tissue × Each column represents one thyroid gland ×× Each column represents an average of 6–10 thyroid glands

Fig 4 Adenoma of a thyroid gland after 16 months of Methylthiouracil treatment Enlargement a) 248 ×, b) 620 ×

crease in oxygen consumption ($P < 0.01$), as shown in Fig 5, whereas the metabolism of glucose and lactate was not increased significantly in these animals (Figs 6 and 7)

The ratio of lactate production to glucose and oxygen uptake respectively (Warburg's quotient) was not found to differ significantly in the two groups (Figs 8 and 9)

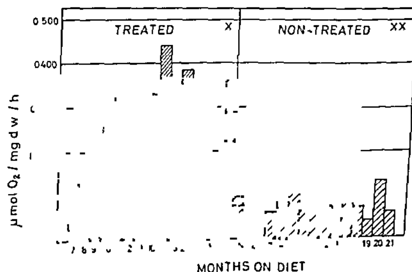


Fig 5 The influence of Methyl thiouracil treatment *on oxygen* consumption of thyroid gland tissue × Each column represents one thyroid gland × × Each column represents an average of *6 10 thyroid glands*

hormone production is not the only factor to play a role in the development of thyroid tumours This is in agreement with observations reported by *Napalkov* (1963)

Another factor to be considered is the iodine content of the fodder, which was 9–10 mcg per gramme According to *Kwa* (1961) this iodine content may have an antagonistic effect on the action of MTU Experiments with a low iodine content have therefore been started

The present studies also indicate that there is no direct correlation between the antihormonal and the goitrogenic effect of the two compounds The effect on endogenous T_4 saturation was the same in Thycapzol and MTU treated mice, but the hyperplasia developed by the latter was about 10 times higher as measured by the weights of the glands

Similarly, any direct correlation between the anti hormonal and the respiratory effect of the two compounds was not found Neither of the two treated groups showed any significant increase in the glycolytic activity, but in contrast to the Thycapzol treated mice (*Jemec* 1970), thyroid glands from the MTU treated groups showed a significant increase in the respiratory activity ($P < 0.01$)

Because of the toxicity of Thycapzol the effect of higher doses on thyroid respiration could not be studied

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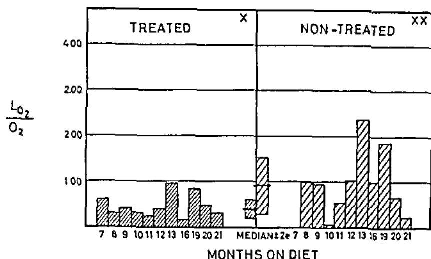


Fig 9 The influence of Methylthiouracil treatment on the ratio of lactate production to oxygen consumption (Warburg's quotient $\frac{L_{O_2}}{O_2}$) in thyroid gland tissue × Each column represents one thyroid gland × × Each column represents an average of 6-10 thyroid glands

Hormone Production

The saturation of the blood with endogenous T_4 was measured in mice treated with Thycapzol (0 05 per cent) and MTU (0 1 per cent) for 2 and 1½ years, respectively The mice were bled from the orbital vein in ether anaesthesia Saturation was evaluated as described by measurements of the capacity of the serum to bind labelled T_3 The results are shown in Fig 10, from which it appears that both Thycapzol and MTU treated mice had a higher capacity to bind T_3, indicating a lower production of endogenous T_4

On the basis of the Wilcoxon test, the results observed in the two treated groups differed significantly from the non-treated control group ($P < 0.01$)

DISCUSSION

The carcinogenic effect in mice which, according to previous reports, may be produced by goitrogenic compounds, such as Methylthiouracil (*Hall & Bielschowsky* 1949, *Bielschowsky et al* 1949), thiourea (*Gorbman* 1946), thiouracil (*Dalton et al* 1946, 1948, 1951, 1951, *Morris* 1951, *Doniach* 1950), propylthiouracil (*Sellers* 1962), urethane (*Uzunov* 1964) was not confirmed by the present study Only two adenomas without any signs of invasive or metastatic growth were observed Various reasons for this discrepancy might be considered

In the present study MTU was added to the drinking water to the point of saturation Previous authors (*Dalton et al* 1948) obtained a 3–5 times higher dose level by the administration of MTU in the fodder Insufficient MTU treatment might thus be one of the reasons for the failure to confirm the carcinogenic effect of MTU However, in the study of the influence of MTU and Thycapzol on serum saturation with T_4 it was found that both compounds were given at doses sufficient to depress thyroid hormone production indicating that inhibition of thyroid

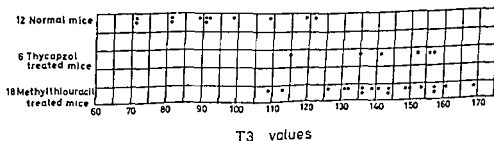


Fig 10 T3 uptake in normal Thycapzol and *MTU treated C3H mice*

Acta path. microbiol scand Section A 79, 553-560, 1971

NECROSIS OF TUMOUR CELLS RELATED TO CIRCULATORY INSUFFICIENCY IN PULMONARY TUMOUR EMBOLISM

KNUT SVANES

The University of Bergen, School of Medicine, the Gade Institute,
Department of Pathology,
Bergen, Norway Head Professor E Waaler M D

Postmortem examination of a woman with epidermoid carcinoma of the cervix uteri revealed numerous tumour emboli in small pulmonary arteries Some arteries were occluded by tumour cells showing extensive necrosis in the central part of the lumen In many arteries groups of tumour cells were present along the vessel wall while the central area was occupied by a partially organized thrombus or connective tissue The tumour cells looked viable near the vessel wall and close to the intravascular connective tissue, while necrotic cells were often found in the centre of the groups of tumour cells The intravascular tumour growth was poor in arteries with extensive perivascular tumour growth The tumour grew better in perivascular lymphatics than in the arteries It was concluded that the observations made in this case supported the hypothesis that necrosis of tumour cells in pulmonary tumour emboli was due, in part at least, to nutritional insufficiency caused by circulatory disturbances produced by the tumour itself

Blood borne tumour emboli are formed by tumour invasion of veins and capillary blood vessels or by *dissemination of tumour cells* via lymph vessels to the veins The size of the emboli may vary from a single tumour cell up to groups large enough to cause fatal pulmonary embolism (*Willis* 1952) It appears to be well established that the majority of embolic cancer cells perish within the blood stream Tumour embolism is not synonymous with metastasis (*Schmidt* 1903, *Willis* 1952) However little is known about the factors which determine the fate of tumour emboli arrested in blood vessels (*Weiss* 1967)

The arrest of tumour emboli in pulmonary arteries causes circulatory disturbances which must be expected to impair the nutrition of *the tumour cells in the emboli* —If the capillary circulation in a tissue becomes inadequate, the cells of the tissue will suffer from insufficient supply of nutrients and an accumulation of metabolites, the more so, the farther away from the vessels they are situated Consequently, if the necrosis of tumour cells in pulmonary tumour emboli were due to circulatory insufficiency, the necrotic cells would be expected to be located further away from the nutritive blood vessels than viable cells The case to be described offered an opportunity to test this hypothesis

MATERIAL AND METHODS

74 year old woman in whom an epidermoid carcinoma of the cervix uteri was diagnosed two years

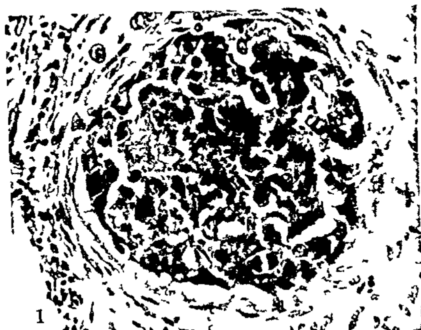
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Requests for reprints should be addressed to dr med *Knut Svanes*, Gades Institutt Avdeling for Patologi Bergen Norway

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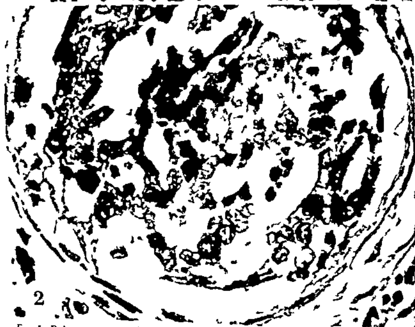


Fig. 1 Pulmonary arteriole (diameter 0.3 mm) occluded by viable and necrotic tumour cells. Most of the viable cells are situated in the peripheral part of the lumen.

Fig. 2 Pulmonary artery (diameter 0.5 mm) occluded by tumour cells. Viable tumour cells are seen at the periphery, necrotic cells in the centre of the lumen.

before death. She was treated with radium followed by surgical extirpation of the uterus, adnexa and pelvic lymph nodes. Postoperative cobalt treatment was given. In the last 4 months she suffered from increasing pain in the right lower extremity. Three months before death she was admitted to hospital because of clinical signs of venous thrombosis in the right leg and for two weeks phenindione was administered. She suffered from chest pain and dysuria and developed paralytic ileus in the final week. Roentgenographic examination of the chest 2 weeks before death showed an infiltration in the left lower lobe. Two days before death the serum non-protein nitrogen was 94 mg per cent.

Autopsy findings. The left pleural cavity contained 200 ml of fluid. The lungs weighed 950 g. A metastasic tumour with a diameter of about 2 cm was found in the left lower lobe. The liver contained a few metastases with diameters of about 1–5 mm. The ureters and renal pelves were dilated. There was diffuse tumour infiltration in the wall of the bladder, causing compression of the lower end of both ureters. Infiltrating tumour masses were present in the vaginal vault and rectum, causing stenosis of the latter. The external iliac veins were surrounded by tumour masses. Metastasis to the first lumbar vertebra was found. The left femoral vein contained a thrombus and a small mural thrombus was present in the right atrium of the heart.

Serial sections were made of one piece of tissue from each lobe of the lungs. The slides were stained with haematoxylin and eosin, elastin van Gieson or Mallory's phosphotungstic acid haematoxylin.

RESULTS

Microscopical examination of the lungs. Six sections from each lobe of the lungs were examined microscopically. The pulmonary tissue was found to be hyperaemic with alveolar oedema in parts. In some areas there was interstitial fibrosis and thick alveolar walls. However, the most significant observations were made in the blood vessels. Numerous small arteries, arterioles and capillaries were found to contain tumour cells. In the present study, special attention was paid to the changes observed in small arteries and larger arterioles (vessel diameters about 700–150 μ). Tumour cells alone or tumour cells together with thrombi or connective tissue were observed in 128 cross sections of arteries and arterioles. On further examination of these vessels the following observations were made.

About six per cent of the vessels were completely occluded by viable and dead tumour cells (Figs 1–2). Most of the viable cells were situated in the periphery, while the degenerate and necrotic cells were found predominantly in the central part of the lumen. There was no ingrowth of connective tissue.

About 25 per cent of the vessels were occluded by tumour cells and thrombus masses. Usually the tumour cells were arranged in groups along the vessel wall while the central part of the lumen was occupied by a thrombus showing more or less organisation (Fig 3). In some arteries a few tumour cells were observed close to the vessel wall covered by a fresh thrombus. Two sections through arterial junctions showed one branch filled with blood and the other occluded by a fresh thrombus containing tumour cells, most of which looked viable and were distributed singly or in small groups in all areas of the lumen.

About 39 per cent of the vessels were occluded by tumour cells and connective tissue, the tumour cells growing along the vessel wall while the central part of the lumen was occupied by connective tissue in which capillary blood vessels were often observed (Figs 4–7). The tumour cells showed a considerable number of mitoses. Viable tumour cells were observed near the vessel wall and often adjacent to the connective tissue while necrotic cells were frequently present in the centre of the columns of tumour cells (Fig 7). The relative amount of tumour and connective tissue varied greatly in the different arteries, most of them showing a picture similar to Fig 6.

Arteries with extensive extravascular tumour growth (about 10 per cent of the tumour containing vessels) showed relatively poor intravascular tumour growth. Some of these arteries contained abundant connective tissue and small groups of tumour cells, others contained some connective tissue and large groups of tumour cells of which a great portion w[illegible] (Figs 8–9).



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Arteries with extensive extravascular tumour growth (about 10 per cent of the tumour containing vessels) showed relatively poor intravascular tumour growth Some of these arteries contained abundant connective tissue and small groups of tumour cells others contained some connective tissue and large groups of tumour cells of which a great portion were necrotic (Figs 8–9)


4
6
5
7



Fig 3 Pulmonary artery (diameter 0 7 mm) A layer of tumour cells is seen along the inner side of the vessel wall The central part of the lumen is occupied by a partially organized thrombus containing some small groups of viable tumour cells

The remaining 20 per cent of vessel cross sections include vessels showing varying and uncharacteristic patterns Some of the smaller arterioles were filled with predominantly viable tumour cells without ingrowth of connective tissue Some vessels contained groups of viable tumour cells separated by connective tissue in the central as well as the peripheral parts of the lumen A few vessels contained tumour emboli surrounded by blood, and a few vessels contained thrombi or connective tissue without tumour cells

The perivascular lymphatics were frequently found to be filled with tumour cells (Fig 4) The number of mitoses was considerably greater and the number of necrotic cells less in the lymphatics than in intravascular groups of tumour cells of comparable size, which indicates that the tumour grew better in the lymphatics than in the blood vessels (Fig 5)

Numerous minute metastases were found, the majority of which were localized in alveolar walls and had probably developed from alveolar capillaries

DISCUSSION

In the present case numerous pulmonary arteries and arterioles were found to be occluded by tumour cells or tumour cells together with thrombi or connective tissue

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Fig 4 Pulmonary artery (diameter 0 6 mm) occluded by tumour cells and connective tissue, with a perivascular lymphatic vessel distended by tumour cells Note the thin wall of the lymphatic and its close relationship to the alveoli

Fig 5 Higher magnification of the lymphatic shown in Fig 4 The tumour shows numerous mitoses and few degenerated cells

Fig 6 Same artery as in Fig 4 Groups of tumour cells are seen near the vessel wall The central part of the lumen is occupied by connective tissue Most of the tumour cells lying close to the vessel wall or in contact with the connective tissue, look viable Some mitoses are present Some necrotic tumour cells are seen in the central area of the groups of tumour cells

Fig 7 Higher magnification of a detail from Fig 6 showing central degeneration and necrosis in a group of tumour cells

A few arteries contained a few tumour cells attached to the vessel wall and covered by fresh thrombus. In many arteries tumour cells were found to grow along the vessel wall while the central area of the lumen was occupied by more or less organized thrombi (Fig 3) Similar observations were made by *Winterbauer et al* (1968) These observations appear to be in accordance with the observation made in rabbits by *Wood* (1964) that circulating tumour cells may stick to the endothelium of the vessels, become surrounded by thrombus and grow —In arteries occluded by tumour and fresh thrombus masses the nutritional problems of the tumour cells will be about the same as in vessels occluded by tumour cells only Nutrition will be best near the vessel wall and this is just where the tumour cells are found to grow best —The ingrowth of blood vessels associated with organisation of the thrombus, provides another nutritional source for the tumour cells and, in organized thrombi, small groups of tumour cells were usually also observed in the central area of the lumen

The lumen of many arteries was occluded by a peripheral zone of tumour cells and a central area of connective tissue (Fig 6) Under such conditions nutrients for the tumour cells might be supplied by the vasa vasorum and the capillaries of the intravascular connective tissue The tumour cells usually looked viable near the vessel wall and close to the central connective tissue while necrotic cells were often observed in the centre of the columns of tumour cells. It is concluded then that in this situation too, the necrotic tumour cells are found at greater distance from the nutritive blood vessels than the viable cells

The intravascular tumour growth was found to be poor in arteries with extensive perivascular tumour infiltration (Fig 8) The extravascular tumour cells get their nutritive substances from branches of the bronchial arteries and from the alveolar capillaries The increasing extravascular consumption of nutrients must be expected to result in a decreased supply of the same substances to the intravascular tumour cells, which may explain the poor growth of the latter.

About 20 per cent of the tumour-containing blood vessels showed changes which provided little information concerning the present hypothesis It should be emphasized, however, that none of the vessels showed changes which could be interpreted as evidence against this hypothesis

The tumour was found to grow better in the perivascular lymphatic vessels than in the arteries, which is in accordance with observations made by *Iwasaki* (1915) It seems reasonable to relate this to the thin walls of the lymphatics and their localization close to the alveoli As a consequence of this, tumour cells in the lymphatics could be expected to receive abundant oxygen from the alveoli and other nutrients from the rich capillary network in the alveolar walls

A minimal tumour embolus arrested in an alveolar capillary would not be expected to cause nutritional disturbances because of the rich capillary network in the alveolar wall and the close contact with the alveolar oxygen This may in part explain the observation repeatedly made that metastases arise more frequently from tumour emboli lodged in the capillaries than from those arrested in the arterioles (*Warren & Gates* 1936, *Coman et al* 1951, *Baserga & Saffiotti* 1955)

It is concluded that the observations made in the present case support the hypothesis that the necrosis of tumour cells in pulmonary tumour emboli is due, in part at least, to nutritional insufficiency caused by circulatory disturbances produced by the tumour and attending thrombosis. On the other hand the present result disagrees with the view expressed by *Iwasaki* (1915) that the necrosis could be due to a deleterious action of blood plasma on the tumour cells

In the present case, organisation of the thrombus covering tumour cells appeared to improve the tumour growth The intravascular necrosis of tumour cells was found to be more extensive in the absence than in the presence of connective tissue, and the tumour cells usually grew well close to the connective

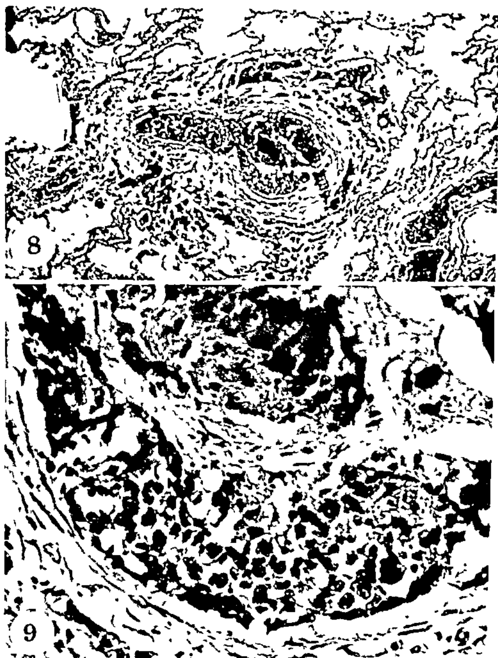


Fig 8 Pulmonary artery (diameter 0·4 mm) occluded by tumour cells and connective tissue Perivascular tumour infiltration

Fig 9 Higher magnification of the artery shown in Fig 8 Extensive necrosis of tumour cells especially in the central area of the lumen The connective tissue looks old and does not grow into the central group of necrotic tumour cells

A few arteries were occluded by tumour cells of which many were necrotic especially in the central part of the lumen (Figs 1–2) Similar observations have been reported by *Schmidt* (1903), *Kikuchi* (1914) and *Iwasaki* (1915) The tumour cells probably represent tumour emboli recently arrested in the pulmonary vessels The emboli interrupt the blood supply to the occluded segments of the vessels and the tumour cells have to get their nutrients and oxygen from the vasa vasorum or other branches of the bronchial arteries surrounding the occluded arteries Consequently, the necrotic tumour cells are found at a greater distance from the nutritive blood vessels than the viable tumour cells

A few arteries contained a few tumour cells attached to the vessel wall and covered by fresh thrombus. In many arteries tumour cells were found to grow along the vessel wall while the central area of the lumen was occupied by more or less organized thrombi (Fig. 3). Similar observations were made by *Winterbauer et al.* (1968). These observations appear to be in accordance with the observation made in rabbits by *Wood* (1964) that circulating tumour cells may stick to the endothelium of the vessels, become surrounded by thrombus and grow.—In arteries occluded by tumour and fresh thrombus masses the nutritional problems of the tumour cells will be about the same as in vessels occluded by tumour cells only. Nutrition will be best near the vessel wall and this is just where the tumour cells are found to grow best.—The ingrowth of blood vessels associated with organisation of the thrombus, provides another nutritional source for the tumour cells and, in organized thrombi, small groups of tumour cells were usually also observed in the central area of the lumen.

The lumen of many arteries was occluded by a peripheral zone of tumour cells and a central area of connective tissue (Fig. 6). Under such conditions nutrients for the tumour cells might be supplied by the vasa vasorum and the capillaries of the intravascular connective tissue. The tumour cells usually looked viable near the vessel wall and close to the central connective tissue while necrotic cells were often observed in the centre of the columns of tumour cells. It is concluded then that in this situation too, the necrotic tumour cells are found at greater distance from the nutritive blood vessels than the viable cells.

The intravascular tumour growth was found to be poor in arteries with extensive perivascular tumour infiltration (Fig. 8). The extravascular tumour cells get their nutritive substances from branches of the bronchial arteries and from the alveolar capillaries. The increasing extravascular consumption of nutrients must be expected to result in a decreased supply of the same substances to the intravascular tumour cells, which may explain the poor growth of the latter.

About 20 per cent of the tumour-containing blood vessels showed changes which provided little information concerning the present hypothesis. It should be emphasized, however, that none of the vessels showed changes which could be interpreted as evidence against this hypothesis.

The tumour was found to grow better in the perivascular lymphatic vessels than in the arteries, which is in accordance with observations made by *Iwasaki* (1915). It seems reasonable to relate this to the thin walls of the lymphatics and their localization close to the alveoli. As a consequence of this, tumour cells in the lymphatics could be expected to receive abundant oxygen from the alveoli and other nutrients from the rich capillary network in the alveolar walls.

A minimal tumour embolus arrested in an alveolar capillary would not be expected to cause nutritional disturbances because of the rich capillary network in the alveolar wall and the close contact with the alveolar oxygen. This may in part explain the observation repeatedly made that metastases arise more frequently from tumour emboli lodged in the capillaries than from those arrested in the arterioles (*Warren & Gates* 1936, *Coman et al.* 1951, *Baserga & Saffiotti* 1955).

It is concluded that the observations made in the present case support the hypothesis that the necrosis of tumour cells in pulmonary tumour emboli is due, in part at least, to nutritional insufficiency caused by circulatory disturbances produced by the tumour and attending thrombosis. On the other hand the present result disagrees with the view expressed by *Iwasaki* (1915) that the necrosis could be due to a deleterious action of blood plasma on the tumour cells.

In the present case, organisation of the thrombus covering tumour cells appeared to improve the tumour growth. The intravascular necrosis of tumour cells was found to be more extensive in the absence than in the presence of connective tissue, and the tumour cells usually grew well close to the connective

tissue This is probably due to the ingrowth of blood vessels associated with organization The observation appears to disagree with the hypothesis that tumour cells are killed by organisation of the thrombus covering the tumour cells (*Schmidt* 1903, *Giertsen* & *Hansen* 1964)

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HISTOLOGY OF THE INDUCTION PHASE OF THE PRIMARY IMMUNE RESPONSE IN LYMPH NODES OF GERMFREE MICE

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Half an hour, 4, 8, 16, 24 hours and 6 days after the injection of chicken red blood cells (CRBC) into the hind foot pads of germfree mice their popliteal lymph nodes were studied in one micron sections and in the electron microscope The untreated germfree lymph node was characterized by the absence of a sinus system and a poor cellularity Mast cell degeneration dilatation of small blood vessels infiltration of the peripheral lymph node parenchyma with polymorphonuclear cells and formation and widening of a subcapsular sinus space were early signs of antigenic stimulation Later, dilatation of high endothelial venules filled up with leucocytes lymphocyte infiltration and lymphocyte degeneration were prominent features of the induction phase Six days after stimulation germinal centre activity had developed

The time interval from administration of antigen until the specific antibody can be detected in serum or tissue is called the induction phase This period is usually considered to be about 24 hours for the primary immune response

There is ample evidence that cellular events taking place during the induction phase are of special importance for the initiation of the primary immune response, and that an intact lymphoid tissue during this early phase is of paramount significance for the development of a normal antibody response Thus damage inflicted upon the lymphoid tissue, e g by administration of cortico steroids or ionizing irradiation will result in severe depression of the immune response when the damage is manifest during the induction phase, but will cause no or only minor reduction if the damage upon the lymphoid tissue is manifest earlier or later (*Taliaferro* 1957, *Berglund* 1965)

In spite of the importance of the induction phase cellular events and histological changes in lymph nodes during this period are largely unknown It has proved difficult to study these early changes in conventional animals. The lymph node is a dynamic structure and is continuously undergoing changes The changes induced by the experimental antigenic stimulus will be superimposed upon these background changes and are difficult to distinguish We therefore thought that lymph nodes of germfree animals would constitute an ideal object of study Although the lymph nodes of germfree animals are also dynamic

Received 24 iii 71

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structures, the background changes are probably reduced to a minimum – lymphoid tissue draining the alimentary tract being excluded – and it can be assumed that no major antigenic stimulus had reached the regional node before the one given by the investigator

For these reasons the popliteal lymph nodes in germfree mice were studied following the administration of heterologous erythrocytes in the foot pads Chicken red blood cells (CRBC) were chosen as antigen, as they are visible in histological sections The lymph nodes were studied in epon embedded 1 γ sections This technique gives excellent details and still offers a good survey of the node (*Söderström* 1967) When further details were desired electron microscopy sections were provided from the same specimens

MATERIAL AND METHODS

Male mice of the NMRI strain, about 2 months old, were used They were born under germfree conditions and kept germfree during the experimental period in a Trexler isolator, model Bornum I Injections and other manipulations were performed in the isolator The germfree status of the isolator was controlled by the procedure outlined by Wagner (1959), before, during and up to one month after the termination of the experiment

The antigen used was CRBC Blood was obtained under sterile conditions and washed three times in sterile saline An eight per cent solution was shunted into the isolator through an atmosphere of per acetic acid vapor The experimental mice received 25 microlitres of the SRBC solution in the foot pad of each hind leg Non injected or saline injected animals served as controls

Animals were removed from the isolator ½ 4 8 16 and 24 hours and 6 days after the antigen injection Under ether anaesthesia the popliteal lymph nodes were dissected out and immediately placed in a 6 per cent solution of glutaric acid After one hour of fixation the organs were post fixed in 2 per cent osmium tetraoxide dehydrated in ethyl alcohol and embedded in epon 812

Serial sections cut at 1 μ with a glass knife were stained with 1 per cent touluidine blue and examined under the light microscope For electron microscopical studies sections were cut on an LKB-ultramicrotome and examined in a Hitachi HS 8 electron microscope

The mice killed 6 days after immunization were bled and the serum haemolysin content measured

Histological sections of CRBC were prepared in the way described above from a sample of the pellet obtained after centrifugation of the chicken blood in order to facilitate the recognition of CRBC in the sections under study

RESULTS

The dose of CRBC used gave a good antibody response in germfree mice the serum haemolysin titre of mice killed 6 days after the injection of the antigen was 3 0 on the average measured as the negative log 2 of the serum dilution giving 50 % haemolysis The titre of non injected controls was 0 2 on the average The popliteal lymph nodes of the animals killed 6 days after immunization were large and contained germinal centres and a well developed medulla with plasma cells

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Figs 1–12 One micron sections (light microscopy) and 400 Å sections (electron microscopy) of popliteal lymph nodes from untreated germfree mice and from germfree mice at different intervals after immunization with chicken red blood cells (CRBC) in the foot pads

Fig 1 Lymph node from an untreated mouse Note the broad subcapsular zone (a) and the post capillary venules with high endothelial cells (arrows) × 180

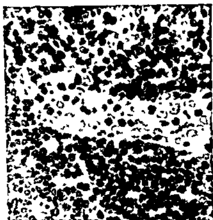
Fig 2 Lymph node from an untreated mouse Lymphocytes in the wall of a postcapillary venule with high endothelial cells × 300

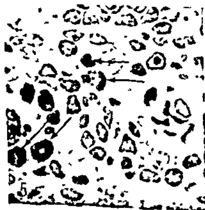
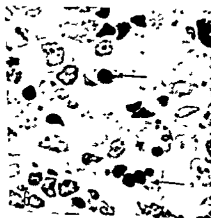
Fig 3 Peripheral part of a lymph node half an hour after immunization Granules from disrupted mast cells are seen all over the section A few intact mast cells are seen (arrows) × 400

Fig 4 Peripheral part of a lymph node 4 hours after immunization CRBC are seen in the developing marginal sinus (arrows) Note the intact mast cells (m) × 500

Fig 5 Peripheral part of a lymph node 4 hours after immunization Polymorphonuclear granulocytes (arrows) in the developing marginal sinus × 750

Fig 6 Peripheral part of a lymph node 8 hours after immunization Formation of a marginal sinus space Note the CRBC localized to the cytoplasm of a macrophage (arrows) × 750



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Histology of Lymph Nodes from Untreated Control Mice

A representative histological section is shown in Fig 1 A most striking feature of the germfree lymph node was the lack of a marginal sinus space In its place a broad subcapsular zone, completely filled with macrophages, reticular cells, mast cells and some lymphocytes was found

A clear-cut cortex and medulla could not be distinguished The outer parts of the lymph node contained small and medium-sized lymphocytes with reticular cells and postcapillary venules with high endothelial cells (H-E venules) The lumen was as a rule not visible in the sections of H-E venules Some lymphocytes could be seen in their walls (Fig 2)

Towards the centre of the lymph node the reticular parenchyma and H-E venules became a more prominent feature The lymphoid cells were few in number here A medullary sinus was not found in untreated germfree lymph nodes

Histology of Regional Lymph Nodes from Immunized Mice

Half an Hour after Immunization

In the popliteal lymph nodes drastic changes have occurred already half an hour after the administration of CRBC (Fig 3) The number of mast cells of the subcapsular zone has increased, based upon semiquantitative histology the increase was tenfold from the control situation Practically all the mast cells had released their granules which were scattered in the tissue At the same time numerous small spaces had developed in the subcapsular zone Some polymorphonuclear granulocytes and small dark lymphocytes had entered the subcapsular area Others were found in the dilatated blood vessels which also contained clots of thrombocytes Only very few CRBC were encountered, they resided in the subcapsular zone

4 Hours after Immunization

The changes in the various popliteal lymph nodes were not fully synchronized In some nodes the number of mast cells in the subcapsular zone has increased even further In all cases the mast cells looked intact The cells occupying the subcapsular area tended to be single and the marginal sinus was thus developing Free mast cell granules were seen, but were scarce CRBC were found in great number in the developing marginal sinus, most of them lying free, (Fig 4), a few being phagocytosed by macrophages

The number of polymorphs in the developing marginal sinus was also greatly increased (Fig 5)

A diffuse infiltration of small lymphocytes has taken place, most conspicuous in the peripheral cortex of the lymph node (Fig 5)

8 Hours after Immunization

The popliteal lymph nodes had increased in size as compared with the controls Along

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Fig 7 Eight hours after immunization CRBC (c) localized to the cytoplasm of a macrophage close to the marginal sinus (n) Nucleus of the macrophage (p) Phagolysosomes × 6000

Fig 8 Peripheral part of a lymph node 8 hours after immunization Infiltration of polymorphonuclear granulocytes in the sheaths surrounding the vessels (arrows) × 400

Fig 9 Eight hours after immunization Part of a polymorphonuclear neutrophil infiltrating the marginal sinus area The dilatation of the perinuclear cisterna (c), the dark cytoplasm and the several lobes of the nucleus (n) are signs of degeneration × 10 000

Fig 10 Lymph node eight hours after immunization A secondary follicle (arrows) is seen in the cortical area × 400

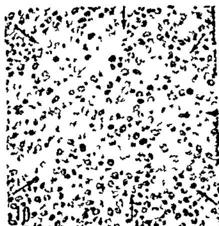
Fig 11 Central part of a lymph node *16 hours* after immunization Note the *small dark lymphocytes* squeezed in between other cells (arrows) × 1000

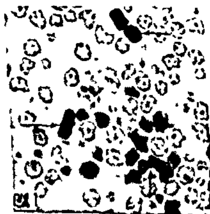
Fig 12 Sixteen hours after immunization *Decaying* lymphocyte with great dilatation of the perinuclear cisterna (c) Note the normal appearance of the neighbouring lymphocytes (l) × 10 000













with the growth and expansion of the lymph node mass, a narrowing of the marginal sinus took place and it became more like a space (Fig 6) CRBC were found in the marginal sinus, but also in the peripheral parts of the parenchyma, most of them were situated in the cytoplasm of macrophages (Fig 6) An electron microscopic picture of ingested CRBC is shown in Fig 7

The number of mast cells and neutrophils were still high in the marginal sinus, many neutrophils were also seen in the sheaths surrounding the vessels (Fig 8) In the electron microscope the polymorphs showed signs of degeneration (Fig 9)

Small lymphocytes appeared in increasing numbers in the endothelial cells of the H E venules and also in the marginal sinus

A structure similar to a secondary follicle was seen in one of the 8 hour specimens (Fig 10)

16 Hours after Immunization

The number of mast cells and granulocytes in the marginal sinus has decreased and approached normal values A few CRBC were still present in the sinus Otherwise no major changes have occurred, except that small dark lymphocytes of irregular shape have appeared centrally in the lymph node (Fig 11) These lymphocytes appeared to be closely packed between other cells and were often situated just outside an H E venule In the electron microscope these lymphocyte appeared to be degenerating (Fig 12)

24 Hours after Immunization

More degenerating lymphocytes of the queer shaped dark variety had accumulated in the interior of the lymph node The H E venules were well developed and had many more lymphocytes in their endothelial cells than in the control situation It was the impression that the cellularity was greater in the centre of the node and not in the periphery as compared with earlier stages Mast cells were present in the marginal sinus, but granulocytes were absent, as in the untreated lymph node Mitoses were not seen in lymphoid cells during the first 24 hours after immunization

DISCUSSION

The popliteal lymph node of a germfree mouse is small and has certain characteristics It lacks the sinus system and instead of a marginal sinus it contains a 50–100 μ subcapsular zone filled with reticular cells, mast cells, macrophages and some lymphocytes

Following antigenic stimulation with CRBC a variety of changes takes place in the regional lymph node already within half an hour The mast cell response was the most conspicuous finding In germfree NMRI mice mast cells were found in the subcapsular zone exclusively, during the first half hour after antigenic stimulation their number increased considerably, and practically all the mast cells had disrupted and released their granules In her study of the mast cell response in conventional mice following injection of soluble protein antigens, *Roberts* (1970) also found accumulation of mast cells, but they were localized to the medullary region and to the interfollicular cortex She also described the degranulation of mast cells, which was such a prominent feature in our ½ hour lymph nodes

It has been argued (*Smith* 1963) that mast cell degranulation can be brought about by non antigenic stimulation In the present study, however, control mice injected with saline did not show changes of the mast cells *Keller* (1966) considered mast cell degranulation to be due to the presence of antigen antibody complexes on the cell surface Evidence for natural antibodies cross reacting with antibodies to CRBC in conventional mice was reported by *Laskov* (1968) Thus it is possible that the dramatic mast cell response in our material was caused by preformed cytophilic antibodies to CRBC

The replication of mast cells is considered to be a slow process (*Asboe Hansen & Levi* 1962, *Miller & Cole* 1968, *Roberts* 1970) So the rapid appearance in our stimulated

lymph nodes of intact mast cells must be due to either the regeneration of granules in persisting cells or to the recruitment of new mast cells

Very rapid changes in the regional lymph node following stimulation with CRBC in conventional guinea pigs was described by Litt (1967) Lysed CRBC were found in the regional lymph node already 5 minutes after the injection in the foot pad

The accumulation of granulocytes was also an early phenomenon, the peak response was 4–8 hours after the administration of antigen A similar observation was made by *Kelly* (1970) The polymorphs in our material – all somewhat damaged as judged by the electron microscopic appearance – were seen in the prospective *marginal sinus* but also inside and just outside vessels They probably enter the node by way of the afferent lymph as well as by the blood stream Their function in the immune reaction against CRBC, however, is not clear

Increase in size – though not quantified – of the regional lymph node was noted from the 8th hour following stimulation, and onwards to the 24th hour Mitotic figures were

[illegible]

lymphocytes some of these were more heavily stained than those already *residing* in the lymph node of the *germfree mouse* *Slonecker* (1969) also found increased cell *numbers in the* regional lymph nodes during the first 24 hour period *following* local injection of pertussis vaccine or sheep red blood cells into conventional rats

The antigen CRBC first appeared in the developing marginal sinus at first as free cells later on ingested by macrophages According to *Bauer* (1967) the capacity of phagocytosing is the same in conventional and germfree animals but the rate of disintegration of the foreign material is slower in macrophages of germfree animals We found that the majority of the foreign erythrocytes were lying freely in the regional lymph node four hours after the injection, but that they were phagocytosed in great numbers after eight hours Electron microscopic investigation of macrophages containing nucleated erythrocytes revealed many active phagolysosomes as indicated in the electron micrograph

Postcapillary venules with high endothelium exist in the unstimulated lymph node of the germfree mouse. Following antigenic stimulation the endothelium became higher and small lymphocytes were found in increasing numbers within the endothelial wall This is in accordance with the description by *Marchesi & Gowans* (1964) and by *Gowans & Knight* (1964) *of the route of recirculation* of small lymphocytes from blood to the lymph node parenchyma *via* high endothelial venules *Kruger* (1968) also stated that H E venules were present in the germfree state. He furthermore described the presence of secondary follicles in untreated animals, and so did *Pollard* (1967) We also found a structure very much like a secondary follicle in the 8 hour series and also six days after antigenic stimulation Of course it is doubtful whether the secondary follicle in the 8 hour-situation can be ascribed to the antigenic stimulation with CRBC

The dark squeezed lymphocytes, which in the electron microscope appear to be dying cells were found in increasing numbers 16 and 24 hours after antigenic stimulation They may have a significant function in the development of the primary immune response, but their function is unknown

In the unstimulated germfree lymph node no *medullary sinus* was found and the marginal sinus was filled with cells It seems safe *to conclude that antigen stimulation is* necessary to the development of the sinus system of the germfree lymph node

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GLOMERULAR LESIONS IN ADULTS WITH THE SCHONLEIN-HENOCH SYNDROME

A Light and Electron Microscopy Study

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Renal biopsy specimens from four adult patients with the Schonlein Henoch syndrome and complicating renal disease were examined by light and electron microscopy By light microscopy three patients showed diffuse hypercellularity of intercapillary cells and hyperplasia of intercapillary cell matrix in almost all glomeruli From twenty to fifty five per cent of all corpuscles from these patients had epithelial crescents in Bowman's capsule In one patient with focal and segmental proliferative changes, but without crescents, focal and segmental thrombosis and necrosis were found Three patients had moderate to severe glomerular infiltration with Polymorphonuclear leucocytes Electron microscopy confirmed the proliferation of intercapillary cells and hyperplasia of intercapillary cell matrix. Hypercellularity of visceral epithelial cells and endothelial cells were not found In the epithelial crescents of Bowman's capsule two cell types could be recognized dark cells and light cells In between these cells basement membrane like material was demonstrated Electron dense deposits were seen in the subendothelial space of the basement membrane, and in the cytoplasm of endothelial cells To a lesser degree, similar deposits were found in the intercapillary region, in the subepithelial space, and in visceral epithelial cells No 'humps' were observed

The Schonlein Henoch syndrome is a generalized vasculitis of unknown aetiology, except for the few cases due to food allergy and drugs (1 14) or to insect bite (4, 27, 29) Among the symptoms from skin, joints, gastro-intestinal tract and kidneys the renal involvement is the most serious complication because of the risk of progressive renal disease Approximately 40 per cent of the patients with this syndrome will develop acute renal disease, 5 per cent of the later will die in acute failure, and from 5 to 38 per cent will develop chronic kidney disease (2, 5, 6, 25, 31, 32) The percutaneous renal biopsy method, in combination with improved light microscopic tecnique, and application of electron- and immunofluorescent microscopy have increased our knowledge of the Schonlein Henoch syndrome, but most of the re-

Received 3 ii 71

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TABLE 1 *Glomerular Lesions in Adults with the Schonlein Henoch Syndrome*

Case no	Male years	No of glomeruli in specimen	Proliferative changes: Focal and segmental	Generalized and diffuse	PMN infiltration	Corpuscles with crescents (in per cent)	Thrombosis	Necrosis
I	56	15	0	+	+	25–55	0	0
II	44	25	0	+	+	20–25	0	+
III	68	14	0	+	0	40–50	0	0
IV	16	14	+	0	+	0	+	+

glomeruli (generalized) and the whole tuft (diffuse) are affected

The dominating histological picture in cases Nos I II III was the presence of epithelial crescents in Bowman s capsule (Fig 1) in from twenty to fifty five per cent of all glomeruli The proliferation of parietal epithelial cells could include tubulus-like formations (Fig 2) In all patients there were proliferation of intercapillary cells and hyperplasia of intercapillary cell matrix (Fig 1) Apparently there was also proliferation of visceral epithelial cells The hypercellularity was focal and segmental only in case



Fig 3 Renal corpuscle with segmental changes in the form of thrombosis of a single vascular loop (lower left In the same area there is *proliferation* of intercapillary cells Silver Methenamine and H & E (× 260)

Fig 4 Renal corpuscle with necrosis and thrombosis in about half of the vascular tuft. Note also the PMN leucocyte infiltration (arrows) PAS and H & E (× 260)

ports are dealing only with cases seen in children In the following we present the results of light- and electron microscopic studies of percutaneous renal biopsy specimens from 4 patients with the Schonlein-Henoch syndrome and complicating renal disease

MATERIALS AND METHODS

Renal biopsy specimens from four men aged 16–68 years were investigated All patients had symptoms from skin, joints, gastro-intestinal tract and kidneys The renal disease was complicated by a nephrotic syndrome Renal biopsy was performed at an average of six weeks from onset of illness Tissue samples for light microscopy were fixed in four per cent buffered formaldehyde, pH 7 0, embedded in Paraplast and cut at two, three and four μ and stained with H&E, PAS and haematoxylin, Silver Methenamine and H&E, Picrosirius, Masson-thrichrome, Lendrum's Fibrin Stain For electron microscopy, tissues were fixed in one per cent osmium-tetroxide dehydrated in acetone and embedded in Vestopal-W Ultrathin sections were stained with Uranyl acetate and Lead citrate The sections were examined with a Hitachi Electron Microscope, Model HU-11E-1

RESULTS

Light Microscopy

The glomerular lesions found in the four patients are shown in Table 1 The changes described as focal and segmental indicate that only few glomeruli in the biopsy are involved (focal) and that the alterations in these affect only part of the vascular tuft (segmental), contrary to lesions described as generalized and diffuse where almost all

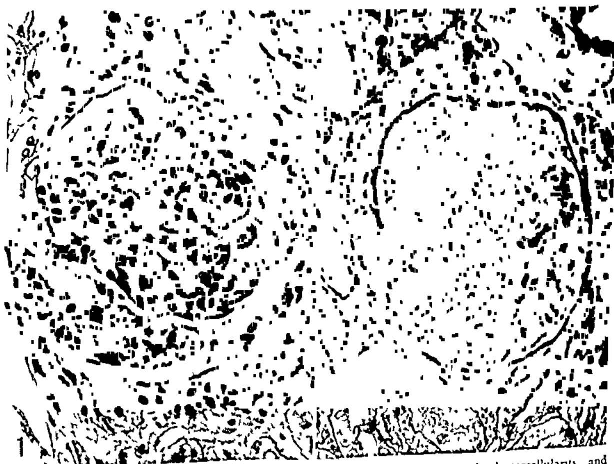


Fig 1 Renal corpuscle with epithelial crescent in Bowman's capsule Note the hypercellularity, and the hyperplasia of intercapillary cell matrix together with the increased lobulation PAS and H&E (× 260)
Fig 2 Bowman's capsule with epithelial proliferation and tubulus like formation (upper left) Silver Methenamine and H & E (× 260)


B
Ep
CL
F
IC
En
D
IC



Fig 5 Renal corpuscle with diffuse hypercellularity of the vascular tuft and segmental necrosis with almost completely closed capillary lumina Silver Methenamine and H & E (× 260)

IV (Fig 3) In this biopsy no crescents could be found The most predominant lesion in case IV was that approximately fifty per cent of all glomeruli were damaged by segmental thrombosis and necrosis of the vascular tuft (Fig 4) Thrombosis was not observed in other biopsies, but case II showed focal and segmental necrosis (Fig 5)

In cases I, II, IV, extensive scarring was seen in a single glomerulus

Three of the four patients had moderate to severe glomerular infiltration with Polymorphonuclear leucocytes (PMN leucocytes)

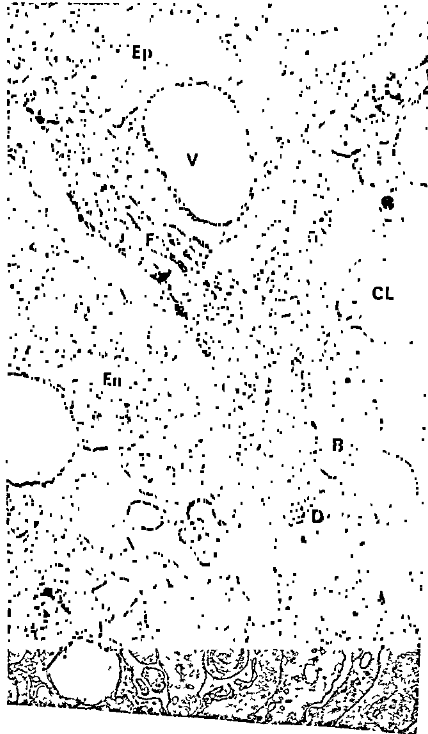
Electron Microscopy

The ultrastructure of the glomeruli examined varied from normal or minimal changes to alterations of serious character A prominent feature was the presence of subendothelial granular and/or linear deposits (Fig 6) with an electron density quite different from that of the basement membrane These deposits were always most pronounced near the intercapillary regions but could be localized to most sites of the circumference of the capillaries, thereby reducing the size of the lumina Granular materials were also observed in invaginations of underlying endothelial cells (Fig 6) and intercapillary cells (Fig 6) Proliferation of endothelial cells could not be demonstrated but their cytoplasm was often swollen and ordinary fenestrations along the periphery of vascular loops was lost (Fig 7) The amount of intracytoplasmic vacuoles was not increased PMN leucocytes were regularly found in capillary lumina as well as in intercapillary regions

The glomerular basement membrane was often seen to be wrinkled and tortuous In biopsies with serious alterations, the membrane could always be found duplicated (Fig 9) and extremely thin (Figs 8, 9) but a break of continuity was observed only in one case In some lobuli, short and irregular basement membrane projections extended into adjacent endothelial and epithelial cytoplasm (Fig 7)

As all patients had a nephrotic syndrome there was a more or less pronounced loss of foot processes (Figs 7, 8, 9) The epithelial cells could appear loosened from the basement membrane (Fig 10) and heavy deposits of granular material (Fig 10) were observed in the subepithelial regions Linear

Fig 6 Glomerular capillary wall with heavy deposits in the subendothelial region (arrow) as well as in invaginations of underlying endothelial cells (double arrow) Deposits (D) can also be demonstrated in the mesangial matrix between intercapillary cells (IC) The external surface of the basement membrane (B) is covered by the foot processes (F) originating from the visceral epithelial cells (Ep) En endothelial cell CL capillary lumen Note the difference in electron density between the deposits and the basement membrane (×



Ep
V
F
CL
En
B
D

TABLE 2 *Glomerular Lesions in Adults with the Schonlein Henoch Syndrome*

Author	No of patients	Normal	Minimal changes	Proliferative: Focal and Segmental	Proliferative: Generalized and diffuse	Proliferative: Crescents	Nephrotic syndrome
Heptinstall & Jockes (13)	6	2	–	4	–	–	–
Ballard et al (5)	14	1	1	1	3	2	7
Panner (24)	1	–	–	1	–	–	–
Vernier et al (19)	1	–	–	–	–	1	–
Lewitt (17)	1	–	–	–	–	1	–
Norkin & Wiener (20)	2	–	–	–	1	1	?
Slama & Habib (28)	11	2	–	4	–	1	–

deposits were regularly found in the cytoplasm adjacent to the basement membrane (*Figs 7, 8, 10*) Granular deposits could also be seen in membrane-limited bodies in epithelial cell cytoplasm (Fig 10) Deposits on the epithelial side of the basement membrane ("humps") were not seen Significant proliferation of visceral epithelial cells were not demonstrated The cytoplasm was often swollen with a content of numerous big vacuoles (*Figs 8, 10*)

In all of the glomeruli examined there were proliferation of the intercapillary cells as well as hyperplasia of the intercapillary matrix (Fig 11) Multiplying intercapillary cells extending along peripheral vascular loops could almost totally occlude capillary lumina Granular deposits were localized to the intercapillary matrix (Fig 6) and to projections from this into intercapillary cells, but the amount was never as pronounced as that found in the subendothelial regions PMN leucocytes could be demonstrated in intercapillary regions

The amount of platelets and fibrin in capillary lumina was sparse The basement membrane of Bowman's capsule could be thickened locally Proliferating parietal epithelial cells (Fig 12) forming crescents were regularly demonstrated In between the proliferating cells, basement membrane-like projections (Fig 13) extended from the capsular basement membrane The cytoplasm of the parietal epithelial cells forming the crescents could be either dark or light (Fig 12) thereby forming two distinct cell types No tubulus like formation of parietal epithelial cells was observed

DISCUSSION

The Schonlein Henoch syndrome is a rare disease in adults for which reason only few studies of its clinical and pathological aspects are available (3, 7, 10, 13, 17 20, 22, 24, 28, 32) The aetiology of this syndrome is still unknown, but many different agents have been suggested as causative Already *Osler* (21) called attention to the correlation between serum disease and this syndrome, and

Fig 7 Part of two glomerular capillary loops In the epithelial cell (*Ep*) with the dilated vacuole (*V*) the foot processes are fused In the cytoplasm near the basement membrane (*B*) deposits (D) are shown The basement membrane is irregular because of projections into adjacent endothelial (*En*) and epithelial cytoplasm The endothelial cytoplasm is locally swollen with loss of fenestrations CL capillary lumen F foot processes (× 20,500)

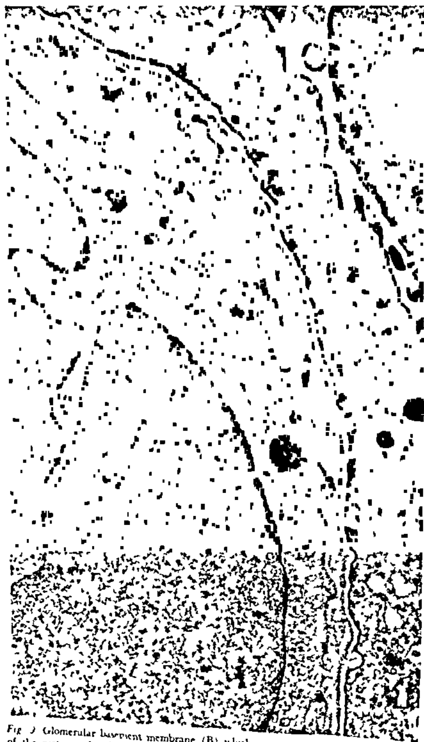


Fig. 3. Glomerular basement membrane (B) which is extremely thin and, at the top of the picture also duplicated. The epithelial cell (Ep) has lost its foot processes. En endothelial cell (× 24 300).

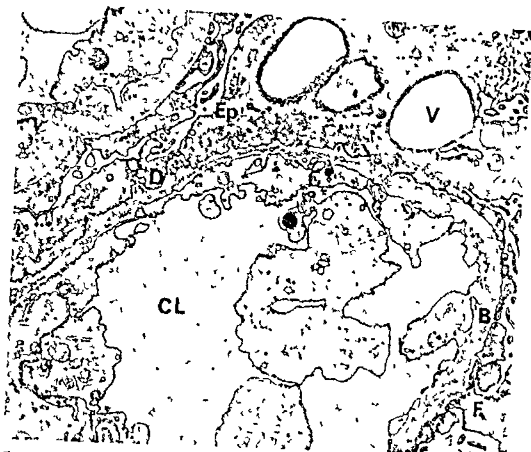


Fig 8 Glomerular capillary with basement *membrane (B)* of decreasing width Most of the epithelial surface of the basement membrane is covered by a continuous sheet of epithelial cytoplasm (Ep) *Only in* the lower right part can foot processes (F) be seen Dense *deposits (D)* are localized to the basal part of the epithelial cytoplasm which also contains large dilated vacuoles (V) *CL* capillary lumen Note the widened *lamina rara externa* (× 11,000)

it has often been *emphasized* that the disease was an allergic reaction, in particular to streptococci *Frodin* (9) *reviewed 23 000* cases *of scarlet* fever but found only four atients with possible Schonlein-Henoch syn-rome *Vernier and associates* (16) observed at the incidence of positive antistrepto-sin O titres was only slightly higher than at to be expected in patients with nonstrep-coccal illness *Ballard et al* (20) found no nnection between the severity of renal sease and presence *or absence of* evidence streptococcal infection The serum com-ement is low in patients with classic acute omerulonephritis (16) but usually normal patients with the Schonlein-Henoch syn ome (12, 33) *Urizar et al* (30) state that though the nephritis of Schonlein Henoch may have an immune pathogenesis, the mechanism probably differs significantly from that of acute poststreptococcal glomerulonephritis, glomerulonephritis of unknown aetiology with or without the nephrotic syndrome, and lupus *glomerulonephritis In the Schonlein* Henoch syndrome they demonstrated prominent mesangial localization of gamma *globulin, complement and fibrinogen, but* less regularly basement membrane oriented granular deposits of immunoglobulins, while the immunoglobulin deposits in acute glomerulonephritis were localized to the epithelial side of the glomerular basement membrane (8, 18, 19) The histopathological picture of the glomeruli differs usually from that of acute poststreptococcal glomerulonephritis, being of a focal and segmental nature (31)

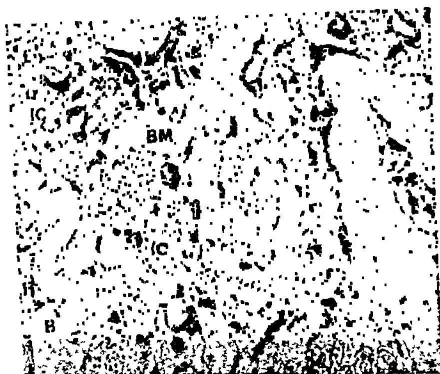


Fig 11 Intercapillary region with intercapillary cells (IC) and intercapillary cell matrix (BM) B glomerular basement membrane F foot processes of visceral epithelial cells En endothelial cell (× 13,000)

The earlier ultrastructural studies of the Schonlein Henoch syndrome are rather few *Regnier & Boussou* (26) found endothelial cell proliferation, focal thickening and splitting of the glomerular basement membrane together with fusion of foot processes where the disease was accompanied by the nephrotic syndrome *Panner* (24), *Falls et al* (7) observed in addition a cell proliferation which proceeded from the intercapillary regions to the peripheral parts of the vascular loops. *Kobayashi et al* (15) and *Urizar et al.* (30) described subendothelial dense deposits *Urizar et al* (30) also demonstrated platelets and fibrin in vascular lumina, and suggested that the renal lesions might be produced from intravascular coagulation

In our study the glomerular proliferative changes were predominantly generalized and diffuse, they were characterized by the presence of crescents in Bowman's capsule. By electron microscopy we did not succeed in demonstrating either endothelial or visceral epithelial cell proliferation The only hypercellularity observed was that of the intercapillary cells and of the parietal epithelial cells As regards the latter, we are in full agreement with *Galle* (4) who maintained that the only glomerular cell types able to proliferate are the parietal epithelial cells and the intercapillary cells In 400 biopsies

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Fig 10 Glomerular capillary loop with extremely thin basement membrane (B) In the subepithelial region a granular deposit (D) and in the neighbouring epithelial cell (Ep) linear deposits (D_1) The epithelial cell contain several membrane-limited bodies with granular deposits (D_2, D_3, D_4) of similar electron density as that of the deposit in the subepithelial region. V dilated vacuole (× 24,300)


D4
D3
V
D1
D2
Ep
D
B
CL

Fig 12 Part of epithelial crescent clearly demonstrating two types of parietal epithelial cells dark cells (DC) and light cells (LC) The basement membrane (B) of Bowman's capsule gives off a small projection, and basement membrane like material (arrow) can be visualized in between the parietal epithelial cells The severely damaged glomerulus (GL) is covered by a continuous layer of visceral epithelial cells (Ep) (× 3 900)

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examined, he found endothelial hyperplasia only in one patient with dysproteinaemia

The occurrence of dark and light cells among proliferating parietal epithelial cells is a new observation The significance of this is unknown

There were no "humps" on the epithelial side of the glomerular basement membrane, but granular deposits were occasionally found between the visceral epithelium and the basement membrane Deposits were always seen to be localized mainly to the subendothelial region and—to a smaller extent—to the intercapillary regions

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Gl
Ep
DC
LC
B

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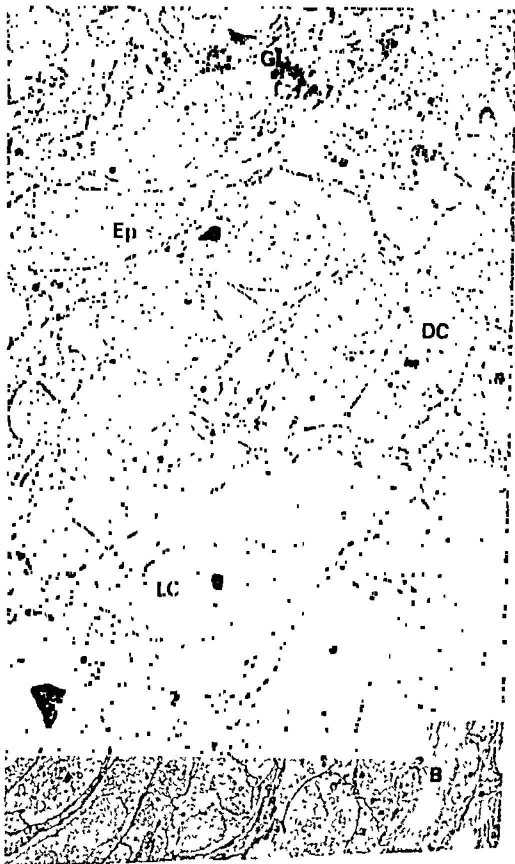

GL
Ep
DC
LC
B

Fig 13 Higher magnification of part of epithelial crescent showing basement membrane (B) of Bowman's capsule and dark (DC) and light (LC) parietal epithelial cells Basement membrane like material (BM) is projecting from the capsular basement membrane (× 13 000)

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DC
LC
B

inmunated before, or, in some experiments, a few days after the inoculation of the Rous virus

MATERIAL AND METHODS

Rats The experimental animals belonged to the inbred rat strain R/F maintained by continuous

Virus The Schmidt Ruppin strain of Rous sarcoma virus (SR RSV) was used in the form of a virus pool prepared according to a simplification of the method of *Bryan et al* (2) as previously described (12) and stored at −70° C. The titre determined according to *Temin and Rubin* (18), was 2×10^5 *FFU/ml*

Tumour cell suspensions for treatment of the experimental animals and lymphoid cell donors were mechanically prepared and irradiated at 0° C by X rays generated at 200 kV, 15 mA and filtered by 1 mm Al The dose was 8000 r

Lymphoid cell preparations for the inoculation of SR RSV treated rats were derived from the *submandibular cervical* axillary, inguinal and retroperitoneal lymph nodes and from the spleen Cell suspensions were prepared by pressing the tissue through a 60 mesh stainless steel screen into Eagle's medium (MEM) The number of viable cells was determined with the trypan blue exclusion test

Lymph node cell (LNC) suspensions for the colony inhibition tests were prepared in the same way without using the spleen

LNC mediated and humoral immunity to the Rous specific TSTA(s) were performed essentially according to *Hellstrom et al* (6 10) as described previously (11) The test target cells were derived from the allogeneic Rous sarcoma RR 6690 induced *in vitro* and serially passaged *in vitro* since two years in Eagle's medium (MEM) with 10 per cent calf serum Control target cells (PBI

the polyoma tumour cells were passaged in Eagle's MEM with 7 per cent foetal calf serum

In the tests for LNC-mediated immunity an appropriate number of tumour cells (500 RR 6690 cells and 200 PBI cells *respectively*), deriving from trypsinized cell suspensions and adjusted to give 50–200 colonies per dish, was added to 60 mm Falcon Plastic Petri dishes. *18 to 24 hours* later the target cells attached to the dishes were exposed to 5×10^4 LNC per dish derived from the experimental and control rats, respectively 4 5 dishes were included for each animal

In the assay for humoral immunity against the Rous-specific TSTA 0 1 ml of a target cell suspension, diluted in Eagle's MEM to contain the appropriate number of tumour cells (1500 or 3000 RR 6690 cells and 600 PBI cells, respectively) was incubated with 0 1 ml serum (from experimental and control animals respectively) for 20 min. at 37° C, after which 0,8 ml guinea pig complement (lyophilized Sclavo Siena), diluted 1 2 was added and the mixture incubated for another 60 min. After dilution with 14 ml of medium the material was divided into three 60 mm Falcon Plastic Petri dishes

Control LNC and sera were derived from rats untreated or pretreated with irradiated isografts of dimethylbenzanthracene induced sarcomas

The dishes were incubated for 3 to 6 days in a humidified 5 per cent CO_2-atmosphere, stained with Giemsa, and the number of colonies counted under low power objective

The per cent reduction of colony numbers obtained with the experimental LNC and sera was calculated from the mean colony numbers of the Petri dishes for the experimental and control LNC and sera, respectively The significance of the numerical reduction was determined according to Student's t test. A few experiments had to be discarded because of infection or unspecific toxic effects on the control target cells.

Experimental design Treatment with irradiated sarcoma isografts during the latency period The rats were inoculated at 4 to 6 *days of age* with 0 2 ml of the SR RSV pool subcutaneously on the back through the right hind leg Each litter was divided into control and experimental groups, the former being treated with irradiated isografts of dimethylbenzanthracene induced (DMBA) sarcoma, the latter with irradiated isografts of Rous sarcoma. Each rat was inoculated four times subcutaneously with the respective cell suspension, *with 7 to 10 days interval* and starting 2 to 4 days after the SR RSV inoculation LNC and sera from these rats were tested for their colony inhibitory effect in CI tests

Treatment with lymphoid cells from immunized donors After SR RSV inoculation at the age of *12 hrs to 8 days* each litter was divided equally into control and experimental groups. One *to 7* days after virus administration control rats were inoculated intraperitoneally with $1,5–2 \times 10^7$ lymphoid cells deriving from *syngeneic donors immunized 2 to 4 times with irradiated* isografts of dimethylbenzanthracene induced *(DMBA)* sarcoma cells Experimental rats were inoculated in parallel with the same number of syngeneic lymphoid cells from donors, immunized in parallel with irradiated Rous sarcoma cells The inoculation of

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IMMUNOLOGICAL INTERFERENCE WITH ROUS SARCOMA VIRUS TUMORIGENESIS IN RATS

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Rats inoculated when newborn with Rous sarcoma virus (SR RSV) were inoculated with irradiated syngeneic Rous tumour cells or with syngeneic lymphoid cells from specifically immunized donors Treatment with irradiated tumour cells gave no demonstrable protection, while administration of specific immune lymphoid cells before and in some experiments a few days after SR RSV inoculation had a protective effect Together with the fact that both tumour negative and tumour positive rats show a high degree of cell mediated and humoral immunity the results indicate that the rapidity with which the cell mediated immunity develops plays an important role for the progressive growth or elimination of the neoplastic cells

The existence of tumour associated transplantation antigens (tumour specific transplantation antigens, TSTA) and the possibility to immunize animals against such antigens provides a basis for prophylaxis and therapy in experimental tumour host model systems A prophylactic effect registerable as a reduced incidence of tumours and/or a prolonged latency period can be achieved by means of an unspecific stimulation of the immune response Thus the development of tumours induced by methylcholanthrene or strontium 90 is delayed by BCG (Bacille Calmette Guerin) administration (15 16) and BCG treatment also results in a reduced incidence and retarded development of adeno 12 virus induced tumours in mice (17) and polyoma tumours in hamsters (13 14) Treatment with Freund s complete adjuvant has a similar effect on adeno 12 tumours in hamsters (1) In the case of viral tumours the cross reactivity between the TSTAs of different tumours induced by the same virus offers the possibility to test the effects of specific prophylactic measures e g treatment with the virus or the specific cellular antigen in question during the latency period Successful results of such experiments in the SV40 system have been reported by several investigators (3 4 5) Other studies have however shown that such preventive effects may depend upon an unspecific BCG effect rather than upon a specific immunization against the TSTAs (17)

This study was intended to clarify whether a specific prophylactic effect in the Rous rat system can be obtained by means of treatment with irradiated Rous tumour cells or with lymphoid cells deriving from donors immunized against such cells A protective effect could be recorded only in animals in which treatment with immune lymphoid cells was

Received 25 iii 71

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lymphoid cells was repeated *four times with 5 to 7* days interval

In one group of rats the first administration of lymphoid cells preceded the SR RSV *inoculation* with 2 to 4 days

After weaning all the rats were marked individually and observed for the development of tumours at the site of SR RSV inoculation and/or haemorrhagic cysts. Doubtful lesions were examined histologically

RESULTS

Effect of treatment with irradiated Rous sarcoma antigen on the frequency of SR RSV-induced lesions The observed cystic lesions and sarcomas in the litters treated with repeated doses of DMBA and Rous tumour antigen respectively, following the SR RSV *inoculation are registered in* Table 1 The *fraction* figures show that neither any reduction in tumour frequency nor any *prolongation* of the latency period is demonstrable in the *Rous* tumour treated group in comparison *to the DMBA tumour treated Nor did un*treated controls (not registered in the table) differ from the two treated groups The frequency of cystic *lesions is* somewhat lower in the Rous group than in the DMBA group, although *the difference is not significant* Thus, *no* clear-cut protective effect, based upon a specific immunization against the Rous TSTA(s) can be demonstrated.

Effect of treatment with irradiated Rous sarcoma antigen on cell-mediated and humoral immunity to the Rous specific TSTA(s) Although no clear-cut effect on the SR RSV induced lesions was apparent, it seemed to be of interest to study whether *the* treatment influenced the LNC mediated and *cytotoxic* immune response As the presence of a primary tumour or cysts might affect the immune reactions (11), the study was concen*trated* upon rats without any lesions The results are collected in Table 2, giving the per cent reduction of colony numbers with LNC *and* sera, respectively, for individual animals. *The table shows* that *7* out of 10 among the negative rats in the control (DMBA treated) group disclose a LNC mediated immunity, *6 out of 10* showing a humoral cytotoxic immune reaction This is *in* good accordance *with the findings in* previous studies of rats *that* did not develop any lesions after SR RSV inoculation *(11) In* the group, treated with irradiated Rous sarcoma antigen, these immune *reactions* are demonstrable at a frequency *about* the same *as that in the* control

TABLE 3 Frequency of Tumours and Cystic Lesions after SR RSV Inoculation into New-Born Rats and Serial Treatment during the Latency Period with Syngeneic Lymphoid Cells from Donors Immunized with Irradiated Isografts of Dimethylbenzanthracene Induced Sarcomas and Rous Sarcomas Respectively

Age at SR RSV inoculation days	Age at first treatment with lymphoid cells (days)	Frequency of lesions* after treatment with lymphoid cells from donors immunized with X irradiated DMBA tumour				X irradiated Rous tumour			
		Cysts	Sarcomas (with or without cysts)	Per cent rats with sarcomas	Mean latency period (weeks)	Cysts	Sarcomas (with or without cysts)	Per cent rats with sarcomas	Mean latency period (weeks)
0 1	2–4	7/21	12/21	57	6	4/20	13/20	65	5
1	8	0/5	5/5	100	5	0/6	6/6	100	5
2 3	7 9	0/9	9/9	100	4	3/13	8/13	62	6
4	11 15	3/17	8/17	47	7	3/17	9/17	53	10
4 6	7 9	3/13	10/13	77	6	1/12	10/12	83	6
5 8	12 15	3/18	12/18	67	8	1/25	19/25	76	10
Total		16/83	56/83	67	6 1	12/93	65/93	70	7,5

* *Fraction figures denote* number of rats with sarcomas and/or cysts over the total number of surviving rats.

TABLE 1 *Frequency of Tumours and Cystic Lesions after SR RSV Inoculation into New Born Rats and Serial Treatment with Irradiated Isografts of Dimethylbenzanthracene Induced Sarcomas and Rous Sarcomas, Respectively, during the Latency Period*

Age at SR RSV inoculation (days)	Age at first immunization (days)	Frequency of lesions* after treatment with X irradiated DMBA tumour: Cysts	Sarcomas (with or without cysts)	Per cent rats with sarcomas	Mean latency period (weeks)	X irradiated Rous tumour: Cysts	Sarcomas (with or without cysts)	Per cent rats with sarcomas	Mean latency period (weeks)
4	6	3/20	8/20	40	13	3/21	8/21	38	10
4–6	8–10	5/8	1/8	13	4	1/8	0/8	0	–
Total		8/28	9/28	32	12	4/29	8/29	28	10

* Fraction figures denote number of rats with sarcomas and/or cysts over the total number of surviving rats

TABLE 2 *Results of Tests for Lymph Node Cell Mediated and Humoral Immunity to the Rous Tumour Associated Transplantation Antigen(s) in Rats Developing No Lesions after Neonatal SR RSV Inoculation, Followed by Serial Treatment with Irradiated Isografts of Dimethylbenzanthracene Induced Sarcomas and Rous Sarcomas, Respectively*

DMBA group: Rat No	Interval from last tumour cell inoculation (weeks)	Per cent reduction* on colony formation with LNC	Serum 1 2+C	Rous group: Rat No	Interval from last tumour cell inoculation (weeks)	Per cent reduction* on colony formation with LNC	Serum 1 2+C
1	12	32**	17**	1	9	4	—13
2	12	4	54***	2	9	n t	9
3	13	9	36***	3	11	2	46***
4	14	n t	29***	4	12	41***	52***
5	14	18**	50***	5	12	29**	—20
6	15	37*	1	6	12	50***	57***
7	15	50*	20*	7	12	31**	31**
8	16	37**	± 0	8	13	11	53***
9	18	—24	— 3	9	15	54**	37**
10	18	21**	—14	10	15	89***	5
11	19	75***	n t	11	15	35***	—12
				12	15	35**	39***
				13	15	76***	—12
				14	15	n t	± 0
				15	15	67**	± 0
				16	15	n t	72***
				17	18	12*	69***
Fraction of rats with significant reduction		7/10	6/10	Fraction of rats with significant reduction		11/14	9/17

* The probabilities that the per cent reductions are due to chance are indicated for each animal $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ Negative figures mean per cent increase in colony numbers compared to controls instead of decrease n t = no test available (technical errors, infections)

has been elicited, while in the SV40 system this immune response develops in due time to interfere with a tumour formation by the more slowly growing or dormant tumour cells.

Another explanation of the discrepant findings in the two systems might be differences in the immunogenicity of the inoculated irradiated tumour material and thus, different effects upon the cell mediated immune reactions and humoral cytotoxic or humoral blocking factors The data presented in Table 2 give no indications of any difference between the experimental rats and the control rats in which *no tumours develop*, as regards the degree of specific cell mediated or humoral cytotoxic immune reactions. In fact, any effect of the repeatedly irradiated *tumour cell inoculations can hardly be expected*, as the large majority of rats, inoculated when newborn with SR RSV but developing no primary tumours show a clear-cut tumour-specific LNC mediated immunity (11) In this respect, tumour negative rats do not differ significantly from rats with established primary tumour As regards humoral cytotoxic activity a stimulating effect of repeated inoculations of irradiated tumour cells in the methylcholanthrene system has been reported (8)

It is possible that treatment with larger antigen doses in the form of unirradiated allografts or initiation of the treatment with tumour antigen before the SR RSV inoculation could have some preventive effect Preliminary experiments in outbred rats were, however, negative (unpublished)

These findings, as well as the fact that primary tumours develop in virtually all *tumour host* systems examined in spite of the presence of a strong cell mediated tumour specific immunity (7–9, 11), indicate that *tolerance* to the TSTA(s) plays no important *rôle in Rous tumorigenesis*. It thus seems plausible that the rapidity with which the immune reactions, especially LNC-mediated, develop might influence the establishment of the tumour cells The *prevention* experiments with immune lymphoid cells were initiated to accomplish a cell-mediated tumour-specific immunity in close temporal proximity to, or *even before*, the appearance of virus-transformed cells. The results indicate that lymphoid cells from specifically immunized donors have a prophylactic effect when given *before*, and in some experiments, a few days after SR RSV inoculation This is in good accordance with the assumption that the rate of development of LNC-mediated immunity can influence the progressive growth or elimination of the neoplastically transformed cells in early tumorigenesis. Studies of the rôle of such time factors are in progress

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TABLE 4 *Frequency of Tumours and Cystic Lesions in Rats induced by SR RSV, Inoculated aft the Beginning of Serial Treatment with Syngeneic Lymphoid Cells from Donors Immunized with I radiated Isografts of Dimethylbenzanthracene Induced Sarcomas and Rous Sarcomas, Respectivel*

Age at first treatment with lymphoid cells (days)	Age at SR RSV inoculation (days)	Frequency of lesions* in rats, treated with lymphoid cells from donors immunized: X irradiated DMBA tumour — Cysts	Sarcomas (with or without cysts)	Per cent rats with sarcomas	Mean latency period (weeks)	X irradiated Rous tumour — Cysts	Sarcomas (with or without cysts)	Per cent rats with sarcomas	M lat pe (w
3	6–7	2/10	6/9	70	27	1/8	2/8	25	1
4	8	0/7	4/7	57	12	0/5	1/5	20	1
Total		2/17	10/16	63	21	1/13	3/13	23	1

* Fraction figures denote number of rats with sarcomas and/or cysts over the total number of surviving rats

group The only difference indicated by the results might be a longer persistence of the humoral cytotoxic reaction in the Rous tumour treated group

Effect of administration of immune lymphoid cells subsequent to SR-RSV inoculation Table 3 illustrates the frequency of SR RSV-induced lesions in rats, virus inoculated at the age of 12 hrs to 8 days and thereafter repeatedly treated with intraperitoneal inoculations of lymphoid cells from syngeneic donors, immunized with irradiated isografts of DMBA and Rous tumours respectively In one group of animals (virus inoculated at 2 to 3 days of age), a reduced frequency of sarcomas in the Rous group is observed that is *just significant at the 5 per cent level* (p=0,049*) In the other groups as well as in the entire material, however no such difference can be shown and the mean latency periods do not differ significantly A slight tendency towards a diminished occurrence of cystic lesions can be observed, although it is far from being significant Also in this set of experiments untreated controls (not shown in the table) did not differ from animals in the DMBA group

Effect of administration of immune lymphoid cell previous to SR RSV inoculation In contrast to the above type of experiments initiation of the treatment with lymphoid cells from specifically immunized syngeneic donors before virus inoculation has a prophylactic effect, manifested as a reduced frequency of Rous sarcomas illustrated in Table 4 Among the rats given lymphoid cells from Rous tumour immunized donors sarcomas developed in 3/13 animals (23 per cent) in contrast to the sarcoma development in 10/16 rats (63 per cent), treated with lymphoid cells from DMBA tumour immunized rats The difference is significant at the 5 per cent level (p=0,034*) The latency periods of tumours did not differ significantly between the groups Cysts developed only in a few rats

DISCUSSION

The failure to demonstrate in the Rous rat system any protective effect of a treatment with homologous tumour antigen subsequent to virus infection is in contrast to the findings in the SV40 system (5) One explanation of the discrepancy may be differences in the time relations between the virus inoculation concerned and the immunizing tumour antigen dose An important point in this connection could be that the latency period of the primary Rous tumours is markedly shorter than that of the SV40 tumours This might imply that the Rous sarcoma cell mass has reached a too large size before the immune response to the inoculations of irradiated tumour antigen

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ULTRASTRUCTURAL STUDIES OF RENAL ARTERIOLAR CHANGES IN ANKYLOSING SPONDYLITIS

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Kidney biopsies from five patients with ankylosing spondylitis were studied electronmicroscopically In four patients renal arteriolar changes were observed. The structure and distribution of the lesions corresponded to the descriptions of renal arteriolar hyalinosis occurring in other conditions. There was nothing to suggest that the deposits originated from the cells, the elastic tissue or the basement membrane of the arteriolar wall Their origin appears likely to be haematogenous Immunological events are possibly involved in producing the vascular lesion

Arteriolar and arterial changes in the kidneys of patients with ankylosing spondylitis were described by *Pasternack et al* (1970) The changes seen in young normotensive people were not associated with any type of treatment and were considered to be in some way a systemic extra-articular manifestation of the disease By immunofluorescence studies, *Linder & Pasternack* (1970) showed that complexes consisting of immunoglobulin and complement were deposited at the basement membranes of the glomeruli and tubuli as well as in the walls of the renal vessels The findings defined more closely the nature of the renal manifestations and suggested that ankylosing spondylitis is associated with immunological processes The possible role of immune complexes was discussed

This study of five patients with ankylosing spondylitis was performed in order to examine the renal arteriolar changes by electron microscopic methods

Received 18 iii 71

PATIENTS AND METHODS

The main clinical data of the patients are presented in Table 1 The diagnosis was based on typical features of the disease early onset, painful anky- [illegible] re, [illegible] od [illegible] u- [illegible] nt [illegible] for both light and electron microscopy Paraffin-embedded sections measuring 3–5 μ were stained with haematoxylin eosin, periodic acid Schiff and silver methenamine In one case (I K.) part of the unfixed tissue was snap-frozen and sectioned with a cryostat for immunohistochemical study (*Linder & Pasternack* 1970).

For electron microscopy the glutaraldehyde-fixed tissue was washed in 7 5 per cent sucrose buffer, postfixed in 2 per cent osmium tetroxide in S-collidin buffer, rapidly dehydrated in alcohol, and embedded in Epon 812

Semi thin sections, 0 5–1 μ thick, were stained with toluidine blue for orientation and selection of suitable blocks by light microscopy Ultra-thin sections were placed on Formvar-coated copper grids, stained with uranyl acetate and lead citrate, and examined with a Siemens Elmiskop I at 60–80 KV

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SMC
D
SMC
END
E
LU
D
SMC
E
D
LU
END
D
D
SMC

TABLE 1 *Main Data of Five Patients with Ankylosing Spondylitis*

Patient	Age years	Sex	Duration of disease years	Drugs prescribed	Blood pressure mmHg	GFR* ml/min/1 73 m²	Urinary findings	Latex/Waaler Rose	Ren arteri chan
I K	21	♂	2	Phenylbutazone	100/70	138	Haematuria	—	+
L V	29	♂	5	Indometacine	125/80	136	—	—	+
B A	32	♂	16	Indometacine Corticosteroids	120/80	149	—	—	+
S N	37	♂	16	Indometacine	125/70	120	—	—	+
E S	48	♂	16	Phenylbutazone Indometacine Corticosteroids	120/85	139	—	—	—

* Glomerular filtration rate

OBSERVATIONS

Light microscopy revealed arteriolar deposits identical with the changes formerly described (*Pasternack et al* 1970) in 4 of the 5 patients (Table 1) The deposits were strongly PAS-positive and were either situated between the intima and the muscle cells or occupied the whole vessel wall Both segmental and circumferential distribution was seen

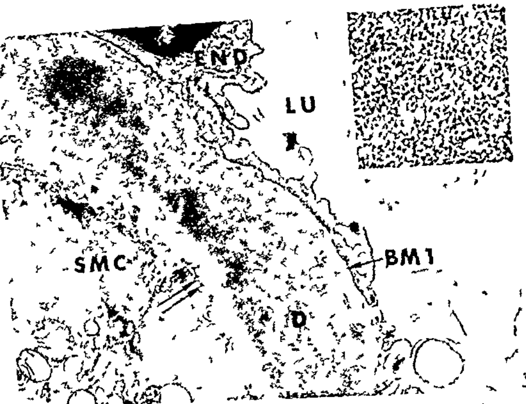
In one case (I K) staining for immunoglobulin and complement showed the presence of IgG and IgM and C'3 at the glomerular and tubular basement membranes and in the arterial and arteriolar walls and deposits A more detailed description of the findings in this case is to be found in a previous paper (*Linder & Pasternack* 1970)

By electron microscopy, various degrees of arteriolar changes were observed in 4 of our 5 cases The impression was gained that the changes occurred predominantly, or maybe even exclusively, in the juxtaglomerular part of the afferent arterioles, the changes often extending into the first intraglomerular continuation of the arteriole and into the hilar parts of Bowman's capsule

The changes consisted of deposits of homogeneous, moderately electron dense finely granular material, which in the cases with minimal changes – which were therefore regarded as the earliest – appeared exclusively in the subendothelium just outside the endothelial basement membrane (Figs 1, 8) In somewhat more advanced cases, the deposits were seen to occupy segmentally the subendothelial space, which to the outside was limited by the elastic elements, or, when these were lacking, by the medial smooth muscle basement membranes (Fig 2) In more extensively developed lesions the deposits extended outwards between the individual medial smooth muscle cells, seemingly pushing and compressing the muscle cells against the periphery of the vessel Thus, in the fully developed lesion, the result was a thickened vessel wall composed for the most part of such deposits, with the medial smooth muscle cells appearing as attenuated layers in the outer-

Fig 1 Minimal deposits in an otherwise normal juxtaglomerular arteriole Subendothelial deposits (D) of granular material are seen in the upper left part of the arteriole and are shown in larger magnification in the inset The endothelial basement membrane (arrows) is intact SMC = medial smooth muscle cells, END – endothelium E erythrocyte LU = arteriolar lumen × 6 900 inset × 51 000

Fig 2 Moderate degree of arteriolar hyalinosis with segmentally distributed space occupying granular deposits (D) A part of medial smooth muscle cell cytoplasm (arrow) is seen enclosed in the larger deposit Otherwise the muscle layer is intact In one place focal deposits (double arrow) are seen also outside the smooth muscle cells Elastic fibres (EL) are evident in some places SMC = medial smooth muscle cells END = endothelium LU = lumen × 4 400



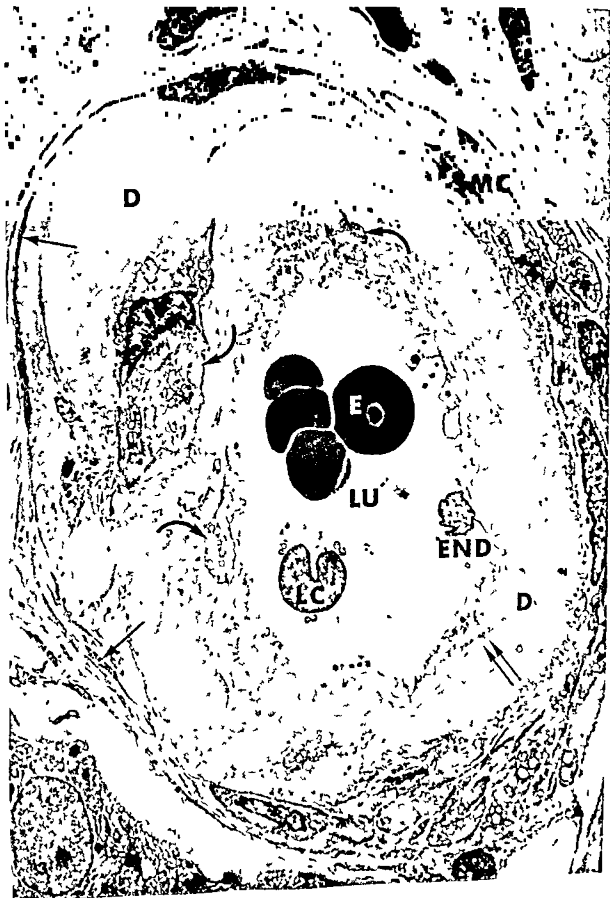
F g 4 Subendothel al space of an arte ole irregularly b oadened by depos ts (D) Ihe granular ature of he depos ts s e dent l own n la ger magn f ca on in the nset Tl e base nent memb anes of tl e endo hel um (BM1) and of l e med al s nooth mu cle cell (doul le a row) can be dent f ed No elast c f b es are seen SMC = n d al smooth n u le cell END endo l el um I U lu nen × 21 000 nset × 64 000

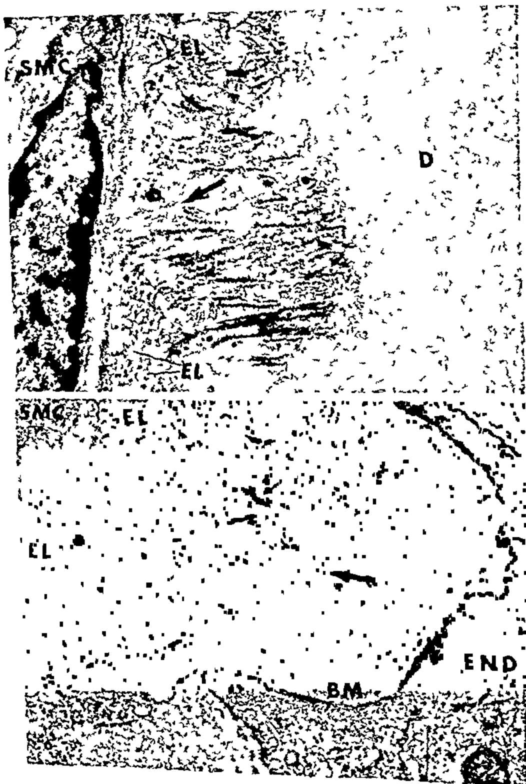
most part of the v all (F g 3) The lumen of the e ren arkably changed vessels vas often r arrowed

The depos ts were usually rather homogeneous be g composed of small granules measuring 100–150 Å n d ameter (F g 4) Not nfrequen ly however the depos ts con ta ned add t onal tructures w th charac ter st cs wl ch son etimes made it poss ble to relate them to adjacent components of the arteriolar wall Thus in the more extensive depos ts parts of the smooth muscle cell cytoplasm v ere often seen enclosed in the deposits (Fig 2 3 & 7) Tl e deposits were usually harply del m ted from the cytoplasm of the smooth muscle cells and no unequivocal transit ons between the two components were observed

I g 3 Afe nt a eriole w h se ere degree of h al no s Tl e depo s D a e repla g mo t of t e wall The med al smoo l muscle ells (SMC) e a enua ed by l e depos ts n ome places ery e mely a g t a ows) Pa s of smoo h n uscle ells curved arrows) a e also enclosed n he depos ts Il e depos s a e crossed by a del ca e n wo k of l asen ent men b ane l ke material d ul le arrow END = endothel um E = ery hroc e l (le oc e LU = lumen × 3 500

The exact relat onsh p between the deposits and the elast c t ssue was more d ff cult to evaluate The granular material often seemed to inf ltrate around elast c f bres (F g 5) and structures resembling n od f ed elast c f bres could then be observed in the depos ts In add t on to structures resembling elastic f bres the outher zone of the deposits occas onally conta ned curved or c rcularly arranged mem


D
MC
E
LU
END
LC
D


SMC
EL
D
EL
SMC
EL
EL
END
BM

brane structures or shorter rods (Figs 5, 6), in which it was sometimes possible to identify a periodicity of about 130 Å (Fig 6) Further, larger granules of various sizes and vesicular structures contributed to give a blurred and irregular appearance to some parts of the outer border of the deposits

The inner border of the deposits, on the other hand, was sharply demarcated from the endothelial basement membrane (Figs 4, 7) Sometimes a very narrow electron lucent space – perhaps a shrinkage artifact – existed between the deposits and the endothelial basement membrane (inset of Fig 1) Structurally, the basement membrane material was distinctly different from the deposits, primarily because it did not exhibit their granular character Larger deposits were usually crossed by a network of duplicated and branching basement membranes which were continuous with the endothelial as well as with the smooth muscular basement membranes (Figs 3, 7) Transitions between the deposits and basement membrane material could not be established with certainty, although in some lesions – regarded as very early ones – material resembling the granular deposits appeared in intimate association with the thickened endothelial basement membranes (Figs 6, 8)

The changes described were not limited to the walls of the juxtaglomerular arterioles In three cases, identical deposits were found in the hilar region of Bowman's capsule (Fig 9), and in one case subendothelial and small intramembranous deposits were also seen in the first intraglomerular part of the arteriole (Fig 10)

In the 10 glomeruli studied (representing all the patients of the series) we noted only a few sporadic deposits in connexion with the peripheral capillary basement membrane One of these sporadic deposits, which were subendothelially located consisted of coarse electron dense granules reminiscent of ferritin particles (Fig 11) Another smaller deposit displayed an amorphous structure, suggesting a lipid composition Except for these sporadic deposits no definite pathological alterations were found in the glomeruli No measurements of the thickness of the capillary basement membranes have been made

DISCUSSION

As was demonstrated in two previous reports (Pasternack et al 1970, Linder & Pasternack 1970), renal arteriolar changes are frequent in young normotensive patients suffering from ankylosing spondylitis Altogether 24 patients, including those of the present series aged 19–54 years (mean age 33 years) were studied In 17 of these (71 per cent), light microscopic renal arteriolar changes were present Exclusion of three cases with amyloidosis gives a frequency of 14/21, corresponding to 67 per cent

Conventional staining of the lesions gave the impression of 'fibrinoid' (Pasternack et al 1970) and these were shown by immunohistochemical study (Linder & Pasternack 1970) to contain immunoglobulin, fibrin and complement The characteristics of the renal arteriolar changes as they appeared in the four patients comprised in this ultrastructural study suggested the term arteriolar hyalinosis (Dustin 1962) This is in fact compatible with both fibrinoid (Dustin 1962 Pardo et al 1966) and immunoglobulin containing de

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Fig 5 Picture showing outer border of a deposit (D) against medial smooth muscle cell (SMC) Elastic elements (EL) though faintly stained are evident near the smooth muscle cell Extending in between the deposits are structures reminiscent of elastic fibres (arrow) and more electron dense fibres rods and larger granules × 24 500

Fig 6 Thickened subendothelial space in juxtaglomerular afferent arteriole Irregular strands of basement membrane material (BM) are seen next to the endothelial cell membrane Surrounded by such material is granular material (thick arrow) thought to represent early deposits More peripherally are many elastic fibres (EL) and curved or circularly arranged membrane structures and rods (small arrows) which show a periodicity of about 130 Å In the upper left corner a small part of medial smooth muscle cell cytoplasm (SMC) END = endot

SMC
EL
D
EL
SMC
EL
EL
D
END
BM

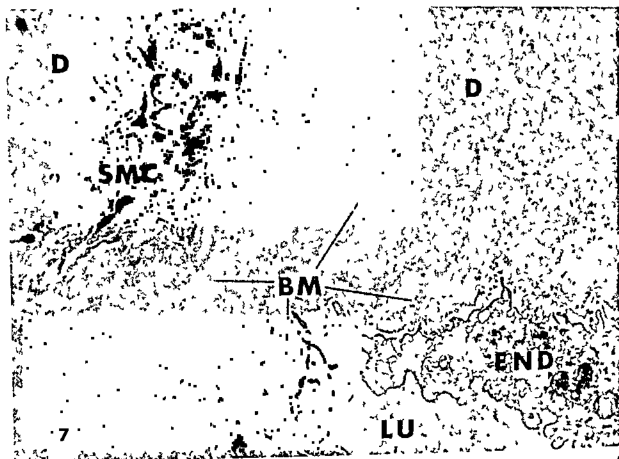
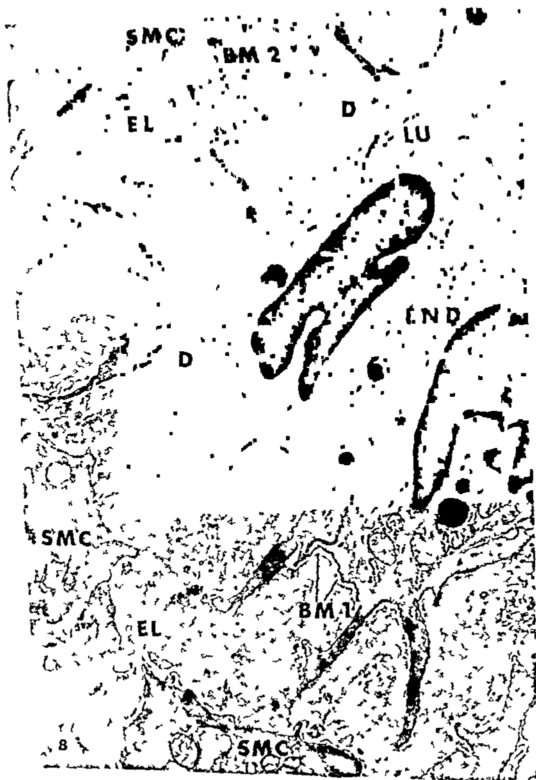


Fig 7 Large deposits (D) crossed by a network of basement membrane material (BM) A part of smooth muscle cell cytoplasm (SMC) is enclosed by deposits END = endothelium, LU = lumen × 14 600

posits (*Dustin* 1962, *Paronetto* 1965) It should be emphasized that the renal arteriolar changes were uniform throughout the series This holds even for the case in which the lesions were observed by light and electron microscopy as well as by immunofluorescence microscopy We thus consider it justified to apply the appellation arteriolar hyalinosis to the renal vascular change frequently seen in ankylosing spondylitis

In electron microscopic studies of normal subjects, *Jacobsen et al* (1966) found no changes of this type However, earlier studies with light microscopic methods had indicated that renal arteriolar hyalinosis exists with a varying frequency *Moritz* & *Oldt* (1937) studied a series of normo and hypertensive subjects over 31 years of age Twelve per cent of the normotensive subjects had hyaline changes and there was a small increase in the frequency with increasing age In contrast, 97 per cent of the hypertensive subjects exhibited changes Among these, too, the changes became more frequent with increasing age *Smith* (1955) found renal arteriolar hyalinosis in nearly half the normotensive autopsy cases he studied, although in nearly all of them the change was slight in extent The frequency in men under 40 was only about 30 per cent In hypertensive individuals, both the fre

Fig 8 Inner part of the wall of a juxtaglomerular arteriole with endothelium (END) to the right and medial smooth muscle cells (SMC) above, below and to the left of the thickened and wrinkled subendothelial space Small collections of granular material (D) are seen near the duplicated endothelial basement membrane (BM1) Elastic fibres (EL) are abundant The endothelial cells contain many organelles including endoplasmic reticulum ribosomes and Golgi apparatus The smooth muscle cell basement membrane (BM2) is well preserved LU = lumen × 15 700


SMC
BM 2
D
EL
LU
END
D
SMC
BM 1
EL
8
SMC

US
EP
D
BC
D
LU
SMC
D
9

JG
END
BC
D
EP
US
LU
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MES
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10

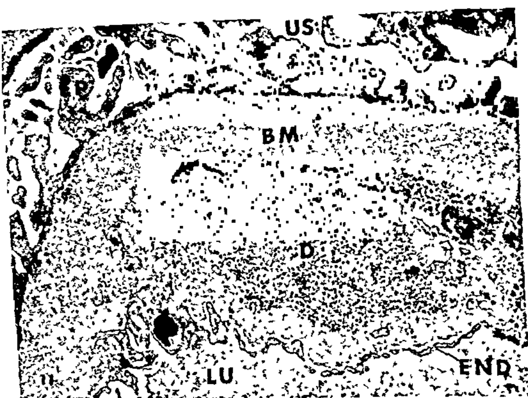


Fig 11 Glomerular capillary basement membrane (BM) with subendothelial (intramembranous) deposit (D) consisting of coarse ringshaped granules reminiscent of ferritin particles US = urinary space, LU = capillary lumen, END = endothelium, EP = podocytes of visceral epithelium × 17 100

Fig 9 Survey picture illustrating a part of a juxtaglomerular arteriole with subendothelial deposits (D) and a part of Bowman's capsule (BC) with identical deposits (D) US = urinary space, EP = parietal epithelium SMC = arteriolar medial smooth muscle cell LU = arteriolar lumen × 4 700

Fig 10 Section through hilar region of a glomerulus with part of the stalk region (MES) in the middle of the picture Subendothelial deposits (D) in the intraglomerularly opening part of the afferent arteriole Bowman's capsule (BC), in this field tapered by epithelial cell podocytes, (double arrow) contains identical deposits (single arrows) LU = arteriolar lumen, CL = capillary lumen, BM = capillary basement membrane, END = arteriolar endothelium, JG = juxtaglomerular granules EP = visceral and parietal epithelium × 5 600

quency and intensity of the changes were greater *Smith* (1955) definitely confirmed the age dependence of the renal arteriolar hyalinosis

Among other conditions frequently associated with renal arteriolar hyalinosis, diabetes mellitus has to be mentioned. *Bell* (1942) found hyaline arteriolar changes to occur in diabetics at a frequency several times that of the controls Again, age dependence was evident In *Smith's* (1955) series, diabetics had a higher frequency of efferent arteriolar hyalinization than normal or hypertensive subjects

When the relatively low mean age of the patients in our series is considered, together with the lack of hypertension and diabetes, it may be concluded that the high frequency of renal arteriolar hyalinosis reflects a pathological condition in some way related to an-

kylosing spondylitis Ultrastructurally, the appearance of the renal arterial hyalinosis in our cases of ankylosing spondylitis exactly corresponded to that described earlier (*Biava et al* 1964, *Fisher et al* 1966, *Pardo et al* 1966, *Salinas-Madrigal et al* 1970) in many of the reports dealing with ageing normotensive subjects as well as with patients suffering from hypertension, diabetes and systemic sclerosis It thus seems a plausible hypothesis that renal arteriolar hyalinosis develops as the ultimate result of some process(es) related to ageing and to various disease states, ankylosing spondylitis being one of them

The derivation of the renal arteriolar hyalin may be discussed in the light of the ultrastructural observations. As has been suggested before (*Biava et al* 1964, *Fisher et al* 1966, *Dustin* 1962, *Pardo et al* 1966), at least two fundamentally different mechanisms are possible The hyalin is either derived from altered tissue in situ or from haematogenous sources

One theory, mainly supported by Muirhead (*Montgomery & Muirhead* 1957) in a series of papers, claimed that the arteriolar hyalin was the result of degenerated smooth muscle Against this it may be argued, that no transitions between the granular deposits and muscle cells occurred On the contrary, the arteriolar hyalin was clearly space-occupying and tended to compress the smooth muscle cells towards the periphery of the vessel

Another theory suggested that arteriolar hyalin is derived from altered basement membrane (*McGee & Ashworth* 1963 *Smith* 1955, *Wiener et al* 1965) However, the amorphous structure of the basement membrane clearly differs from the granular appearance of arteriolar hyalin Indeed, in our preparations the basement membrane was usually well preserved and no definite transitions between it and the hyalin were observed

It has also been suggested that the arteriolar hyalin might be derived from the elastic tissue (*Pardo et al* 1966, *Fisher et al* 1966) Although definite transitions between elastic fibres and the deposits were not seen, the possibility of an elastic origin cannot be excluded

The presence of gammaglobulin and fibrin within the hyaline deposits (*Paronetto* 1965, *Burkholder* 1965) has been taken as evidence of the haematogenous origin of the hyalin The findings of ferritin particles (*Fisher et al* 1966) further supported the haematogenous theory which was formerly favoured by many authors (e g *Zollinger* 1950, *Baker & Schkoff* 1952, *Still & Hill*, 1959) As far as experimental hypertension is concerned, the works of *Giese* (1964) clearly documented the passage of micro particles into the vessel wall The exact mechanism by which the deposition of haematogenous substances takes place in the various other conditions associated with arteriolar hyalinosis is not known Although this remains obscure even in the case of ankylosing spondylitis, some of our observations support the theory of a haematogenous origin

Previous immunohistochemical study showed that immune mechanisms are involved in the renal lesions of ankylosing spondylitis (*Linder & Pasternack* 1970) That the deposits contained immunoglobulin and fibrinogen also indicated that renal arteriolar hyalinosis in ankylosing spondylitis, as in other conditions, is at least partly of haematogenous origin The size of the granules in the deposits corresponds rather closely to previously reported findings (*Biava et al* 1964 *Fisher et al* 1966) and is not incompatible with globular proteins of haematogenous origin The possibility exists that the immunological mechanisms mentioned above play a pathogenetic role by damaging the arterioles which then permit a higher than normal degree of passage of fluids through the endothelium

Deposits having exactly the appearance of arteriolar hyalin were seen in Bowman's capsule Such deposits have been observed in cases of diabetes (*Salinas Madrigal et al* 1970) and experimental monocrotaline induced nephropathy (*Carstens & Allen* 1970) At this site a smooth muscle or elastic tissue origin of the deposits is excluded Lack of transition forms between the deposits and the basement membrane makes their origin from this material equally unlikely It thus appears that the deposits must either result from de-

position of filtered substances or be composed of insoluble substances transported from elsewhere, but there is no evidence for either suggestion

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PRIMARY SARCOMA OF THE HEART

A case report

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Primary heart tumours are extremely rare and have apparently never been reported in Sweden At autopsy, we have had the opportunity of seeing a sarcoma of the heart with certain features of angiosarcoma In this case the clinical symptoms had been blurred by a coexisting heart defect and thereby precluded demonstration of any relation between the cardiac symptoms and the tumour Neither were laboratory data helpful in assessing the age of the tumour

A primary, malignant heart tumour is a rarity No case seems to have been reported from the Scandinavian countries Therefore the following case report might be of some interest

CASE REPORT

Historical A retired male teacher, born in 1888, was admitted to Malmo General Hospital in 1965 because of right sided hemiplegia and aphasia His condition improved somewhat, but he remained crippled and spent the rest of his life in the department for chronic diseases He died in June 1970 at the age of 82 years

His cardiac symptoms began about a decade before his death with signs of congestive cardiac failure For years a systolic murmur of grade III had been heard with *p m* over apex in the 4th intercostal space During the last 18 months he had attacks of arrythmia with periods of tachycardia probably auricular fibrillation with irregular blocking and auricular flutter Roentgenograms taken during the previous five years up to 4 months before death showed gross left ventricular enlargement, but no signs of a tumour Neither were the electrocardiograms of any aid in estimating the age of the tumour The last month before his death he had spells of low blood pressure combined with shock like symptoms

Laboratory data LSR was normal until Sept *1969*, after which it steadily rose to reach a peak of 102 mm on 28th May 1970 The W B C was normal Electrophoresis in March 1970 produced no evidence suggesting a malignant neoplasm

AUTOPSY

Gross findings The body was that of a cachectic old man The heart was enlarged (910 g) with marked left sided ventricular hypertrophy The atrial septum harboured a fist sized tumour The lesion was of a rather meaty consistence It was light grey yellow and showed spots and patches of dark bleeding It had broken through the endocardium in the left atrium The mitral valves were matted together and rigid with calcifications and the mitral orifice consisted of only a narrow slit This seemed to have caused stenosis as well as insufficiency The left ventricle was both hypertrophic and dilated (Fig 1)

Macroscopic metastases were found only superficially in the lower lobe of the left lung which contained a tumour $1^1/_2$ cm in diameter The cut surface of the tumour was a bluish red An old cystic softening nearly 1 cm in diameter was seen in the left cerebral hemisphere No signs of arteriosclerosis in the cerebrum were seen

Microscopic findings The tumour consisted of sheets of fusiform cells with elongated nuclei arranged parallel to each other and in interlacking

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Fig 1 The left side of the heart is exposed The tumour is seen bulging into the left atrium The thickened valves and cordae of the mitral orifice is seen



Fig 2 Histological picture of the tumour with polymorphous spindle cells htx-e × 300

bundles The picture was that of a highly malignant tumour with hyperchromatic polymorphic nuclei with abundant mitoses and invasive growth There were necrotic and haemorrhagic foci (Fig 2) In sections stained for reticulin fibres with a pattern of small vessels were occasionally seen to surrounding tumour cells (Fig 3) This was the only finding suggesting an angiosarcoma in an otherwise anaplastic tumour

Sections stained with Mc Manus and Heidenhain's stains were negative thus giving no supports for a diagnosis of rhabdomyosarcoma

DISCUSSION

This is the first case of malignant primary heart tumour seen at the University Institution of Pathology Malmo where some 17 000 autopsies have been performed from 1955 through 1970 (=0 006 per cent)

Straus & *Merliss* (4) estimated the incidence of primary heart tumours at 0 0017 per cent *i e* in 17 out of one million autopsies



Fig 3 Reticulin fibres surrounding tumour cells sometimes resembling small vessels Gridely reticulin × 460

This estimate was based on a review of autopsy experience in the United States from 1939 to 1942 This is much lower than that previously estimated by *Benjamin* (1) who gave 0 03 per cent of 40 000 autopsies

About 25 per cent of these tumours are considered malignant (3) Altogether 177 cases of malignant primary heart tumours were on record by the end of 1959

According to *Glancy et al* (2), angiosarcoma, though rare, may be the commonest primary malignant tumour of the heart Its frequency is roughly the same as that of rhabdomyosarcoma of the heart and probably higher than that of cardiac fibrosarcoma, reticulumcellsarcoma or lymphosarcoma In 1968 they were able to trace 40 cases of primary cardiac angiosarcoma and to add one their own

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CORRELATION BETWEEN TUBULES AND CAPILLARIES AND SIZE OF INTERSTITIAL SPACE IN THE FUNCTIONING RAT KIDNEY

Influence of Different Types of Preparation

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In rat kidneys the early *post mortem* changes of the cortical tubules and of the peritubular capillaries were investigated *after different forms of preparation*. *In the instantly frozen, freeze dried* tissue, a systematic, correlated orientation of the tubules in the renal cortex was found. The main axis of the tubules was orientated radially out to the surface of the *kidney, and the corresponding peritubular capillaries were* orientated parallel to the tubules. The total interstitial volume in the kidney cortex was found quantitatively significant (21 per cent), including the capillary volume (7 per cent). This was compared to the *conventional fixed tissue,* in which the total interstitial volume was 9 per cent, and in which the capillaries were not readily recognized. *In instantly frozen, freeze substituted tissue* slight artificial changes were found, but the parallel orientation of the tubules and the capillaries could still be recognized. *In freeze substituted tissue, frozen 2 minutes after the removal of the kidney and in conventionally fixed tissue,* the parallel, radial orientation of the tubules and capillaries was distorted.

Following the disruption of the blood supply to the kidney, pronounced qualitative and quantitative morphological changes in the kidney cortex will occur rapidly (*Hanssen* 1960). As the organization of the nephron and its vessels may be concealed by the early *post mortem* changes of the tissue, a systematic investigation of the morphology in the rat kidney cortex has been made by use of modern cryo-preparation of the tissue (*Faarup* 1965, 1967).

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MATERIALS AND METHODS

In 8 white, male, anaesthetized rats of 180–250 g the left kidney was removed and instantly frozen, followed by either freeze drying or freeze substitution in alcohol. In 2 rats the right kidney was freezesubstituted 2 minutes after the kidney was removed from the animal, and in 4 of the rats the right kidney was conventionally fixed.

Group I. Preparation of Freeze Dried, Osmic Acid-Stained Sections of the Instantly Frozen Kidneys

In 4 laparotomized rats the left kidney was removed and instantly frozen in isopentane, cooled by liquid nitrogen to about −160° C. In a cryostat 4–6 micron sections were cut *tangentially to the* surface of the kidney, at the level of the superficial nephrons in the cortex (Fig. 1, section A), as well

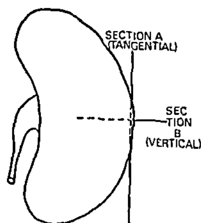


Fig 1 Schematic drawing showing the histological sections used for comparison of the sections from the freeze dried, freeze substituted and fixed kidney cortex
Section A Tangential section superficially in the cortex
Section B Vertical section in the kidney

Fig 2a Freeze dried cryostat section, stained by osmic acid vapour, from instantly frozen tissue The section is made vertically in the kidney tissue The proximal tubules are seen with open lumen (PT) and these as well as the distal tubules (DT) are mainly vertically orientated in the section *The intertubular capillaries* are found to have a parallel orientation with the tubules A well developed interstitial tissue (I) is present in this tissue preparation The arrow in lower left corner *points radially out to* the surface of the kidney and thus indicates the orientation of the section in the kidney cortex (× 820)
Fig 2b Freeze dried cryostat section, stained by osmic acid vapour from instantly frozen tissue The section is made tangential to the surface of the kidney It is shown that the proximal *tubules (PT) as* well as the distal tubules before (asc H) and after (DT) the macula densa are chiefly cross sectioned in this orientation of the section The same applies to the intertubular capillaries *(CAP) In the first* segment of the proximal tubule (PT_1) the brush border is longer and the lumen smaller than in the next segment of the tubule (PT_2) I Interstitial tissue At X *fracture-lines are found* in the section due to the cutting in the cryostat (× 820)

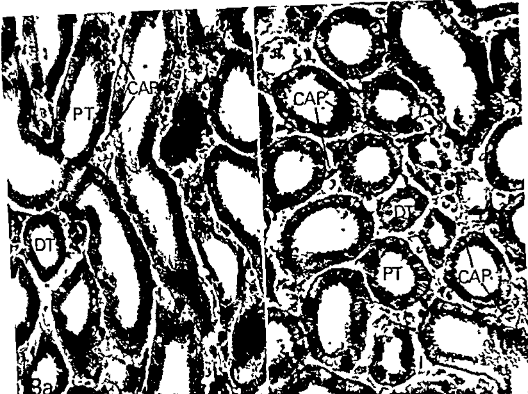


Fig 3a Instantly frozen, freeze substituted tissue The section is made vertically as in Fig 2a and shows that the tubules as well as the capillaries are principally orientated as in Fig 2a although the orientation is somewhat less pronounced than in the freeze-dried tissue
Abbreviations as in Fig 2 The arrows points to the surface of the kidney (PAS stain, × 820)
Fig 3b Instantly frozen, freeze substituted tissue The section is made tangentially to the surface of the kidney as in Fig 2b The tubules and the capillaries are principally orientated as in Fig 2b
Abbreviation as in Fig 2 (PAS-stain, × 820)

as vertically to the surface (Fig 1, section B) The sections were freeze-dried at −20° C placed close to osmic acid crystals in a desiccator (*Faarup 1965*) In this way the sections were stained by the osmic acid vapour during the dehydration and thus the different tubules were easily identified at microscopy (Figs 2a and 2b) The freeze dried sections were finally mounted in paraffin oil and the cover glass sealed with DPX

Group II Preparation of Freeze-Substituted Sections of the Instantly Frozen Kidneys

In 4 laparotomized rats the left kidney was

ing to *Petri* (1968) and *Faarup* & *Petri* (1969)

Group III Preparation of Freeze-Substituted Sections from Kidneys with Delayed Freezing

In 2 rats from Group II the right kidney was frozen 2 minutes after removel from the animal The kidneys were otherwise prepared as in Group II

Group IV Conventional Fixation of Kidneys

For comparison the right kidney of 2 animals from Group I as well as 2 from Group II were Helly fixed and inbedded in paraffin wax The sections were cut as described in Group I (Fig 1) and PAS stained as described in Group II

Technique for Quantitative Estimation of Early Post Mortem Changes in the Kidney Cortex

A The *superficial* nephrons from the kidneys in Group I–IV were used for the measurements per

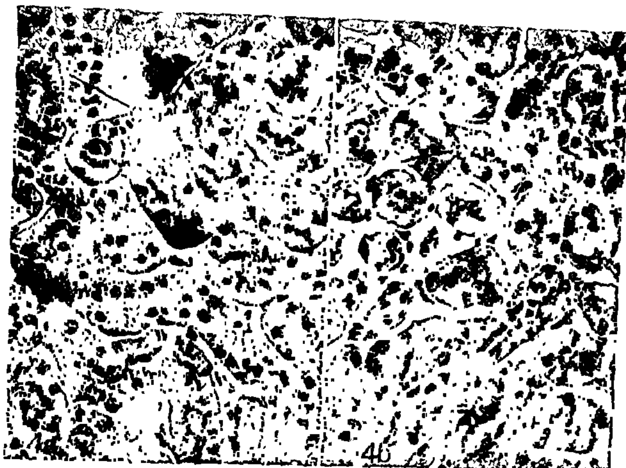


Fig 4a Freeze-substituted tissue from kidney with delayed freezing (2 minutes) Vertical orientation of the section as in Fig 2a. The proximal tubules are occluded and the peritubular capillaries are mostly difficult to identify in the section and contain few or no erythrocytes The vertical orientation of the tubules is difficult to identify

A Arteriole, abbreviations otherwise as in Fig. 2 The arrow points to the surface of the kidney (PAS stain, × 820)

Fig 4b. Freeze-substituted tissue from kidney with delayed freezing (2 minutes) The section is made tangentially to the kidney as in Fig 2b The cross-sectioning of the tubules is difficult to identify in this preparation, and many capillaries are unidentifiable. The interstitial space is partly preserved Abbreviations as in Fig 2 (PAS-stain, × 820)

formed, as the freezing of the kidney will occur earliest in this area of the tissue. In photographs of horizontal and of vertical sections the external diameter of the proximal and distal tubules as well as the capillaries was measured By comparison of the differently prepared kidneys (freeze-dried, freeze-substituted, delayed freeze substituted and fixed tissue, see Group I–IV) systematic *post mortem* changes of the orientation of tubules and capillaries could be evaluated (*cf* Fig 2–5)

B In tangetial sections from 4 kidneys in Groups I, II and IV, which were identical to those used for measurement of the diameter of the structures, an evaluation of the volume of the tubular fraction, and of the vascular fraction of the kidney cortex was made (Fig 11) The volumes were measured by planimetry in micrographs of the tissue as previously used by *Bing* & *Faarup* (1965)

RESULTS

I. Instantly Frozen, Freeze-Dried Tissue

In vertical sections of the cortical tissue from 4 kidneys the tubules were found, very constantly, parallel orientated, as the longitudinal axis of the tubules was pointing radially against the surface of the kidney (Fig 2a) In accordance with this finding, corresponding tangentially sectioned tubules were cross-sectioned (Fig 2b)

Measurements of the diameters of the

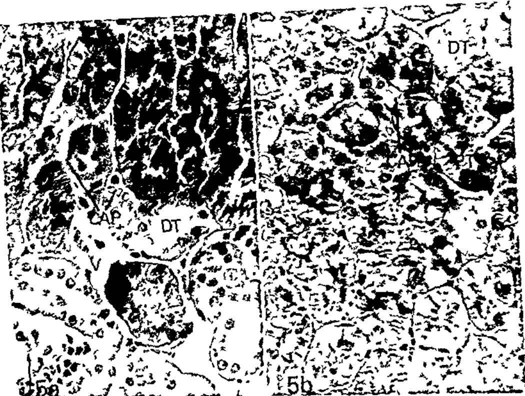


Fig 5a Helly fixed tissue The sect on is ma le … m of the tubules is … the section and the … b Opposite to Fig … ose of the delayed … ud … uote iations as in Fig 2 (PAS stain × 820)

tubules (Fig 6) in vertical sections *showed* that the length of the tubules varied *much* and was significantly greater than the *width* In tangential sections the *two* diameters of *the* tubules were very similar (Fig 6)

By analogous *measurements of the diameters of the peritubular capillaries in* the vertical and tangential *sections* a similar pattern was found (*Fig* 7) as the intertubular capillaries *were* chiefly orientated radially to the surface of the kidney and parallel to the tubules (Figs 2a and 2b)

Mean values of the tubular and capillary diameters in the freeze dried kidneys are shown in Figs 8 and 9 The *uniform* radial orientation of the loops of the proximal tubules was found to be equal in the kidneys investigated As regards the distal tubules, a similar orientation of the loops *was present*, *although* in only 2 of the kidneys investigated a *sufficient* number of distal tubules *were* present in the *vertical* sections.

The mean diameter of the *peritubular* capillaries in the tangential *sections* is seen in Fig 10

Instantly Frozen, Freeze Substituted Tissue

In the PAS stained sections of the cortical tissue *from* 4 kidneys which *were* instantly *frozen* and freeze substituted in alcohol, the tubules were principally orientated as in the freeze dried tissue with the longitudinal axis

TUBULES

width in micron

length in micron

Fig 6 The tubular diameters measured in freeze dried cryostat sections from an instantly frozen kidney In the sections tangentially to the surface of the kidney (○) the diameters measured (*length* and *width* in the figure) are fairly identical In the vertical sections (□) the tubular length is systematically much greater than the width

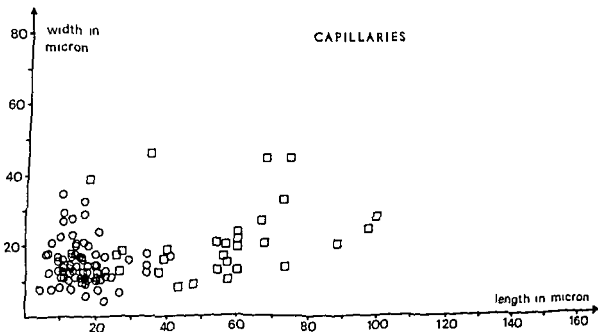


Fig 7 The diameters of the peritubular capillaries measured in freeze dried cryostat section from an instantly frozen kidney In the sections tangentially to the surface of the kidney (○) the diameters measured are fairly identical In the vertical sections the length of the capillaries (□) is systematically much greater than the width (*cf Fig 6*)



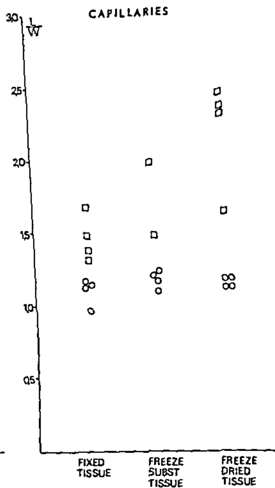


Fig 8 Mean values for the ratio between length and width ($\frac{L}{W}$) of the tubules in tangential (○) and in vertical (□) sections of the kidney in instantly freeze-dried instantly freeze substituted and in fixed tissue

It is seen that in the tangential sections the mean diameter in the two directions is very similar independent of the preparation used However in the vertical sections the tubules are longest in the freeze-dried tissue somewhat shorter in the freeze substituted tissue and shortest although recognizable in the fixed tissue demonstrating that a wrinkling of the tubules will take place during the fixation and freeze-substitution The artificial changes comprises the proximal tubules (open symbols) as well as the distal tubules (filled symbols)

Fig 9 Mean values for ratio between length and width ($\frac{L}{W}$) of the peritubular capillaries in tangential and vertical sections of the kidney expressed as in Fig 8 The pattern of the capillary changes during the freezesubstitution and during the fixation is idential to that of the tubular changes (Fig 8) Concerning the fixed tissue, both the early *post mortem* changes and the fixation will account for the changes in the ratio

pointing radially against the surface of the kidney (Figs. 3a and b) In 2 of the kidneys, *vertical* sections were investigable and in 4 kidneys, tangential sections were investigated with respect to the tubular and capillary diameters. In the vertical sections, the length of the tubules and of the capillaries was found to be slightly shorter than in the freeze dried tissue (Figs 8 and 9) As in the latter, the orientation of the distal tubules was found to vary more than that of the proximal tubules.

In 3 of the 4 kidneys in which the diameter

of the capillaries was measured in the tangential sections, this diameter was significantly larger in the freeze-substituted sections than in the corresponding freeze dried sections (Fig 10)

III Delayed Frozen, Freeze-Substituted Tissue

In the tangential and vertical sections of freeze substituted tissue which was frozen 2 minutes after the removal of the kidney from the animal, the proximal tubules were occluded and no obvious systematic orientation of the tubules or the capillaries was present The peritubular capillaries were mostly difficult to identify in the sections, partly because of the low amount of erythrocytes in the lumen of the vessels (Figs 4a and b) as compared to the instantly frozen tissue (*cf* Figs 2 and 3) The tubular and capillary diameter was not measured

IV Conventionally Fixed Tissue

In the tangential and the vertical PAS stained sections of the conventionally fixed rat kidneys no systematic, regular orientation of the tubules or the peritubular capillaries was attained (Figs 5a and b), in accordance to the morphology of the kidneys from Group III, which were 2 minutes delayed before freezing (Figs 4a and b) As in the latter, the tubules were occluded, and in the vertical sections, they presented a similar wrinkled appearance Accordingly, the peritubular capillaries were oftenly unindentifiable with small lumen and containing few or no erythrocytes The interstitium was scanty, and often it was difficult to separate the intertubular blood capillaries from the connective tissue or lymphatic space

The Relative Volume of the Interstitium the Peritubular Capillaries and the Tubules in the Instantly Freeze-Dried Tissue, the Instantly Freeze-Substituted Tissue and in the Conventionally Fixed Tissue

Tangential sections from 4 kidneys in Group I, II and IV were used for assessment by planimetry of the interstitial, capillary and tubular volume in the superficial part of the kidney cortex. In the instantly freeze dried tissue, an average interstitial volume of 14 per cent and an average volume of the capillaries of 7 per cent was obtained In the instantly freeze substituted tissue the capillary volume was 12 per cent with an interstitial volume of 14 per cent the latter being identical to the of the freeze dried tissue The "concentration" of erythrocytes in the capillaries was significantly less in the freeze substituted tissue as was the case in the freeze-dried tissue in accordance to the different diameter of the vessels in the two preparations (Fig 10)

In the fixed tissue, the total capillary and interstitial volume averaged 9 per cent, due to the collapse of the peritubular capillaries as well as the interstitium (Figs 5a and b)

DISCUSSION

Walker & Oliver (1941) and *Wirtz Gottschalk & Mylle* (1955, 1956) found by vital microscopy of the surface of the functioning kidney that the tubules were open, and that the proximal tubules occluded when the circulation of blood through the kidney was stopped, most of the ultrafiltrate of the tubules being displaced to the blood These morphological changes have been confirmed and extended in careful investigations on freeze substituted tissue by *Hanssen* (1960), who observed that the *post mortem* changes decribed took place very rapidly and that only a sparse amount of erythrocytes was present in the peritubular capillaries in kidneys which were frozen with a delay of 5 minutes

The aim of the present study was to demonstrate the most reliable method by which to prepare kidney tissue, thus demonstrating the structure in the functioning kidney This result was best obtained by use of instantly freezing of the tissue combined with freeze drying of thin cryostat sections stained by osmic acid vapour during the freeze drying procedure In this way the artificial

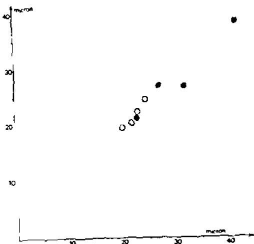


Fig 10 Mean values for the diameter of the peri tubular capillaries in two directions in tangential sections from instantly frozen freeze-dried (○) and from instantly frozen freeze substituted tissue (●) In the figure the mean diameter of the capillaries is greater in 3 of the 4 freeze substituted kidneys as compared to the 4 freeze-dried kidneys complementary to the shrinkage of the tubules during the alcohol substitution (*cf* Figs 2b and 3b)

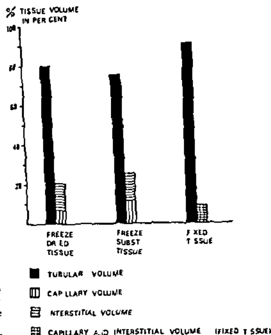


Fig 11 The tubular, the capillary and the interstitial volume in per cent from freeze-dried, freeze substituted and fixed tissue as calculated by planimetry from tangential sections of the kidneys. *In the freeze-dried and in the freeze-substituted tissue* a significant rather equal interstitium is present, although the capillary volume in the alcohol-substituted tissue is nearly twice that in the freeze dried tissue This is caused by the tubular shrinkage during the substitution process by which the capillaries are compensatorily dilated (*cf* Figs 2b 3b and 10)

changes of the tissue, caused by contamination with water are totally omitted In such a preparation the tubules and the capillaries were found more parallel orientated than in the other preparations applied (Figs 2, 6, 7, 8 and 9) A somewhat less reliable result was obtained if instantly frozen freeze substituted tissue was used as a slight wrinkling of the tubules and of the capillaries would occur (Figs 3a and b) and the peritubular capillaries would be somewhat dilated complementary to the changes of the tubules caused by the substitution (Figs 10 and 11)

The first and the second segment of the proximal tubule were easily distinguished in the freeze dried sections (Fig 2b) As found by *Maunsbach* (1966) in glutaraldehyde perfused kidneys the luminal diameter was largest in the second segment

More severe artifical changes of the tubules and of the capillaries were present in the delayed freeze substituted and in the fixed tissue (Figs 4 5 8 and 9) *Accordingly, the* regularity in the orientation of the peritubular capillary was similarly obscured in microangiographied formalin fixed tissue of the renal cortex (*Ljungqvist* & *Lagergren* 1962)

The *interstitial space* of 14 per cent in the *freeze dried tissue diminished appreciably in* the fixed tissue (Fig 11) The occurrence of *a quantitatively considerable interstitial* space in the functioning kidney can be of importance as a *place of formation of* angiotensin, corresponding to the finding of both renin and angiotensin *in the* renal lymph (*Lever* & *Peart* 1962 *Skinner et al* 1963, *Bailie et al* 1971) thereby influencing the function of the nephron (*Leyssac* 1966, *Thurau* 1969), and thus the interstitial space around the tu-

bules and the capillaries may permit that an essential part of the influence of the renin-angiotensin system upon the kidney function is due to extravascular formation of angiotensin. Provided the angiotensinase-activity in the interstitial space is correspondingly low, the angiotensin thus formed will have optimal conditions for a regulatory influence upon the nephron.

The present finding of a correlated orientation of tubules and capillaries in the renal cortex are in accordance with the hypothesis of *Steinhausen* (1970) concerning the existence of a cortical counter-current system between capillaries and tubules. Neither his studies nor those here presented allow an evaluation of a possible function of the anastomoses between the intertubular capillaries from adjacent nephrons, which have been previously described (*e.g. Morison* 1926).

Note added in proof. The quantitative volume changes of the renal cortex has been discussed in *The cortical volume of the normal human kidney* by *V. Hegedus and P. Faarup* (Acta radiol. scand. in press).

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DEGREE OF MALIGNANCY OF CANCER INVOLVING THE CERVIX UTERI, JUDGED ON THE BASIS OF CLINICAL STAGE, HISTOLOGY, SIZE OF NUCLEI, AND CONTENT OF DNA

S Nodskov Pedersen

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The aim of the present study has been to correlate the degree of malignancy of tumours involving the cervix uteri expressed by a 5-year survival rate, with histological morphology, nuclear size and content of DNA The series comprised 117 patients DNA-contents were determined histochemically by U V absorption, the nuclear sizes being determined by planimetric measurements The clinical stages and 5-year survival rates were found to be correlated and furthermore malignant tissue from patients with cervical tumours was found to present increased contents of DNA in nuclei of the tumour cells as well as significantly enlarged cellular nuclei A correlation between histological morphology and degrees of malignancy was not observed and our study fails to provide a basis on which to confirm the findings obtained by other authors for instance, that prognostic data might be obtainable on the basis of the relative DNA content and/or nuclear sizes in malignant tissue

Cells in malignant tissue possess certain properties by which they deviate from the corresponding normal cells first and foremost, growth of such cells may be autonomous and invasive and they may have a metastasizing capacity by which they cause the death of the host organism These properties manifest themselves in the relationship of cells to the host organism

Furthermore most tumours in man are characterized by a histologically demonstrable dedifferentiation and certain intracellular changes such as increases in chromosomal numbers, content of DNA, and size of nuclei.

Quite a long series of studies are available in the literature according to which many tumours in man have been found to be aneuploid and also that the DNA values were elevated in most of the malignant tumours (1-5, 10, 14, 15, 20-22, 25, 30, 31, 35, 41)

The content of DNA in malignant tissue from patients with gynaecological cancer has been examined, for instance, by *Atkin et al* (2, 3, 4), *Grundmann et al* (11), *Reid et al* (30), *Sandritter et al* (30, 31), and *Valeri et al* (36) In the case of cancer involving the cervix uteri, patients in the series studied

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by *Atkin et al* were classified, according to content of DNA and size of nuclei, into two groups, namely one in which tumours were hyperdiploid and one in which they were hypotetraploid These authors found that the prognosis of patients belonging in the hyperdiploid group was essentially less favourable than the prognosis of patients belonging in the hypotetraploid group, whether judged on the basis of determinations of DNA content or measurement of nuclear size

In the present study of patients with cervical cancer it has been attempted to have the clinical stage the degree of histological differentiation, size of nuclei, and content of DNA correlated with a 5-year survival rate

MATERIAL

The series comprised a total of 117 patients namely 102 patients with cancer involving the cervix uteri and 15 normal women The malignant material was derived from patients who were admitted to the Radiumcentre in Copenhagen in order to have radiation therapy The material was collected in the period from the Spring of 1964 until the Summer of 1965 and follow ups have covered five years The normal material was obtained from patients without malignant disease who were admitted to the gynaecological department of Bispebjerg Hospital

In tissue specimens from these 117 patients, the size of nuclei was determined in 108 cases, the content of DNA being determined in 50 cases in 40 cases the content of DNA as well as the size

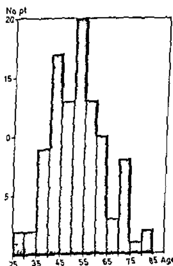


Fig 1 Distribution of age in the malignant material

of nuclei were determined, although different biopsies from one and the same patient served for the latter determination Cases here described as carcinoma in situ are histopathologically verified carcinomata in situ in patients presenting clinically demonstrable signs of cervical cancer A few patients have been excluded either because they died of intercurrent diseases, or because of failure during the preparation of tissue specimens

The distribution according to age of the 117 cases of malignant tumors appears from Fig 1 This age distribution is identical with that observed by other authors in cases of cervical cancer (18) The average age of normal patients comprised in the present study was 43 years

METHODS

Material to be used for estimations of DNA content and nuclear size was obtained by biopsy from the periphery of the tumour in untreated patients The samples were taken by a skilled gynaecologist The material was processed immediately after removal The biopsy was divided into two portions one to be used for histology and determination of nuclear size and one to be used for DNA estimation the latter was cut into small pieces measuring less than 1 mm across The material was fixed in Carnoys solution (alcohol 99 per cent chloroform glacial acetic acid 6 3 1) for about one hour, depending on the size of the sample With a view to DNA estimations, the tissue specimens were placed in a water bath at 60°C and hydrolysed in 1 N HCl for 12 5 minutes After hydrolysis the samples were stained according to Feulgen Schiff (27)

In order to further disintegrate the tissue from its intercellular substance the tissue was placed in 20 per cent glacial acetic acid for 5 minutes after staining and cut with knives into small pieces that were placed between two slides and squashed to have the nuclei spread Finally the slides were mounted

The DNA values were estimated by a Leitz microspectrophotometer The experimental set up was a modification of the Pollister apparatus (26)

Wave lengths of 546 nanometer were used for the estimations In every slide 50 tumour interphase nuclei were measured The total nuclear DNA content was calculated on the basis of the extinction value of the central part of the nucleus and the diameter of the nucleus it was expressed in arbitrary units as described by *Leuchtenberger* (19) The tissue samples to be used for histology and nuclear size measurements were stained with haematoxylin eosin embedded in paraffin and cut into sections about 10 μ thick The nuclear size has been estimated by planimetry using a horizontally placed Zeiss microscope A HBO lamp served as light source The microscopic pic

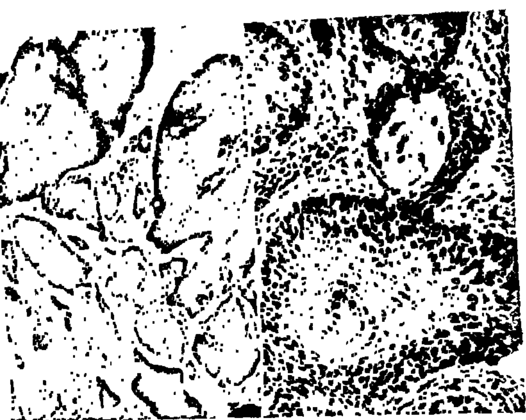


Fig 2 Highly differentiated tumor of the uterine cervix

ture was magnified 1200 times and projected to a plane table One hundred nuclei, selected at random in each slide, were measured planimetrically, each nucleus being measured three times, and the average calculated

The histologically demonstrable differentiation of the tumours was estimated in collaboration with an experienced pathologist who was unacquainted with the clinical course Only patients in whom a diagnosis of spinocellular carcinoma had been established by microscopy were included in the material A few patients were excluded from the material because of poor preparation of the samples

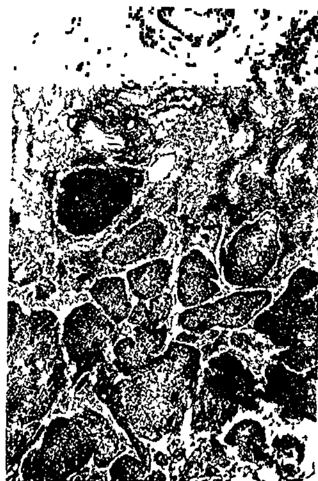
According to the histopathology our malignant material has been divided into carcinoma in situ, and highly moderately and low differentiated, invasively growing tumours From a histopathological point of view carcinoma in situ is a lesion in which the entire thickness of the layer of squamous epithelium is replaced by cells which microscopically are indistinguishable from those of frank invasive cancer and in which loss of stratification is complete, but invasive penetration of the basement membrane is absent

Findings in highly differentiated carcinoma include large abnormal cells, epithelial pearls, keratinization, and a low mitosis index (Fig 2) In moderately differentiated tumours findings include pleomorphic cells, absence of epithelial pearls occasional keratinization, and a moderately high mitosis index (Fig 3) In low differentiated carcinomata, pleomorphic cells are numerous and small basophil cells are not infrequent, the ratio nucleus to cell and the mitosis index are high (Fig 4)

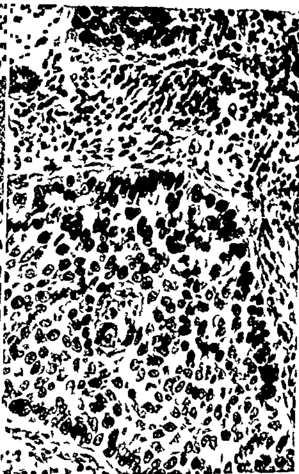
The clinical classification into stages was made by a skilled gynaecologist while patients were anaesthetized Differentiation between clinical stages I, II, III, and IV, is in accordance with the international standard adopted in 1961 (16)

As mentioned above, the term histopathological carcinoma in situ applies in the present study to cases in which cervical cancer was clinically demonstrable

The material has been statistically analysed by



H & E × 40



H & E × 100

Fig 3 Moderately differentiated tumor of the uterine cervix

Students t test unless otherwise stated as for instance in the case of some of the tables

RESULTS

The results obtained appear from the following tables and figures

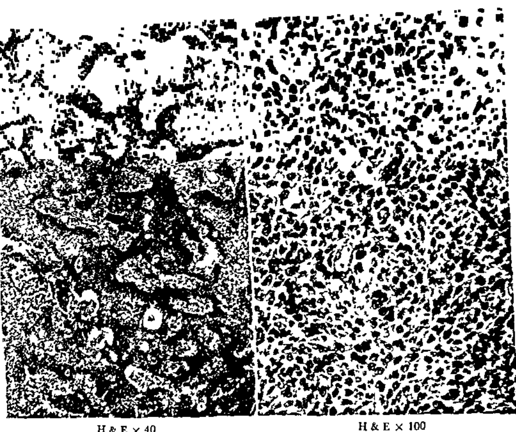
In *Table 1* the clinical stage is correlated with a 5 year survival rate Development of the lesion from stage I into stage IV brings about that the 5 year survival rate is reduced namely from 90 per cent to 11 per cent The distribution according to age of patients who either died or in whom tumours recurred appears from *Fig 5* according to which the age distribution did not deviate from that in the entire material A few patients who died of intercurrent diseases are excluded

It is apparent from *Table 2* that any clear cut correlation between histological grades and clinical stages could not be established *Table 3* shows that the difference in 5 year survival rates was not statistically significant in the various histological groups

The correlation between clinical stage and nuclear size appears from *Table 4* The difference in nuclear sizes in normal and malignant tissue is significant while such difference is not in evidence if the individual clinically malignant stages were compared

TABLE 1 *Clinical Stage 5 Years Survival*

	No pt alive	Dead or rec	Total
St I	18 (90%)	2 (10%)	20
St II	23 (57%)	18 (43%)	41
St III	12 (46%)	14 (54%)	26
St IV	1 (11%)	8 (89%)	9



H & E × 40 H & E × 100

Fig 4 Low differentiated tumor of the uterine cervix

TABLE 2 *Degree of Differentiation – Clinical Stage*

Degree of differentiation	Total	Clinical stage I	II	III	IV
Highly	12	2	4	6	0
Moderately	60	14	25	14	7
Low	15	2	9	3	1

It can be seen from *Table 5* that nuclear sizes which deviate significantly from those observed in normal tissue and are of the same order of magnitude as those encountered at stages of invasive growth are demonstrable at the early histopathological stage carcinoma-in-situ Furthermore, the table shows that sizes of nuclei are not dependent on degrees of differentiation The average size of nuclei in patients surviving for five years and of those in patients who either died or tumours recurred within five years, is not significantly different (*Table 6*). *Fig 6* depicts diagrammatically the average size of nuclei in the individual patients A vague shifting to the left towards lower values is noticed among patients who either died or in whom tumours recurred If patients were classified into two groups (*Table 7*) according to values above or below 52 5 arbitrary

TABLE 3 *Degree of Differentiation – 5 Years Survival*

	No pt alive	Dead or rec	Total
Highly	6 (55%)	5 (45%)	11
Moderately	31 (54%)	26 (46%)	57
Low	7 (47%)	8 (53%)	15

TABLE 4 *Clinical Stage – Nuclear Size*

Clinical stage	x	n	s	2 e
Normal	27.02	15	3.23	1.67
St I	50.39	17	7.41	3.60
St II	49.88	41	8.95	2.79
St III	50.54	24	6.63	2.71
St IV	47.51	9	4.54	3.03

The nuclear size is given in arbitrary units

TABLE 5 *Degree of Differentiation – Nuclear Size*

	x	n	s	2 e
Normal	27.02	15	3.23	1.67
Carcinoma in situ	50.69	8	7.69	5.44
Highly diff invas c	49.97	11	6.19	3.73
Moderately diff invas c	50.00	58	8.04	2.11
Low diff invas c	49.30	13	8.22	4.56

The nuclear size is given in arbitrary units

units, the relative number of survivors in whom tumours did not recur (72 per cent) would be seen to range a little higher in the former than in the latter group although the difference judged on the basis of the X^2 test ($P > 0.05$), was not significant In the succeeding two Tables (*8 and 9*), the clinical stage and histological degree of differentiation respectively, are compared with the average content of DNA per nuclei, specified by arbitrary units Significant differences between normal and malignant tissue are in evidence By comparison of the individual clinical stages, the difference between stage II and stage IV is found to be significant ($0.01 > P > 0.001$) while the other clinical stages do not differ significantly, particularly not stages I and IV, probably owing to a wide scatter The highest DNA values are observed during stage IV and in the lowest differentiated tumours In analogy with features concerning nuclear sizes, values at the histologically premalignant stage carcinoma in situ are of the same order of magnitude as those observed at malignant stages, but the

TABLE 6 *5 Years Survival – Nuclear Size*

	x	n	s	2 e
No pt alive	51.28	49	8.57	2.45
Dead or rec	47.58	38	5.95	1.93

The nuclear size is given in arbitrary units

three degrees of differentiation do not differ significantly *Table 10* represents a comparison between 5 year survival rates and DNA content Any characteristic pattern is not apparent from this table, however, or from *Fig 7* in which the average DNA content in the individual patients is histogrammatically represented

The succeeding two figures (*Figs 8 and 9*) show examples of DNA karyograms of various tumours, indicated by arbitrary units The karyogram seen uppermost in *Fig 8* is obtained from a normal patient in whom the diploid modal values were within normal range The other DNA karyograms illustrate how the DNA content in tumours may vary *from diploid to aneuploid, heteroploid and* polyploid values The clinical stages of the individual tumours are plotted together with the histologically demonstrable degree of differentiation and time of survival of patients

TABLE 7 *5 Years Survival Nuclear Size*

	No pt alive	Dead or rec
Nuclear size <52.5	28 (48%)	30 (52%)
Nuclear size >52.5	21 (72%)	8 (28%)

X_2 test with Yates correction.

TABLE 8 *Clinical Stage – DNA*

Clinical stage	x	n*	s	2 e
Normal	*1.40*	*100*	*0.36*	0.07
St I	3.30	11	1.47	0.89
St II	3.13	19	0.80	0.37
St III	3.71	14	*0.76*	*0.41*
St IV	4.42	5	1.25	1.12

* n = number of patients in each group with exception of the normal group in which n is the number of cell nuclei from two patients

TABLE 9 *Degree of Differentiation – DNA*

Clinical stage	x	n*	s	2 e
Normal	1 40	100	0 36	0 07
Carcinoma in s tu	3 55	4	0 88	0 88
Highly diff invas c	3 18	6	0 79	0 64
Moderately diff invas c	3 32	28	0 94	0 36
Low diff invas c	3 94	8	1 70	1 21

* See Table 8

TABLE 10 *5 Years Survival – DNA*

	x	n	s	2 e
No pt alive	3 57	21	1 04	0 46
Dead or rec	3 34	24	1 18	0 48

The DNA-content like the foregoing tables in arbitrary units

The last karyogram illustrates a tumour in which the modal DNA values are in the hyperdiploid as well as in the tetraploid region

DISCUSSION

According to previous investigations, it has been generally accepted that the prognosis in cases of cervical cancer depends on a series

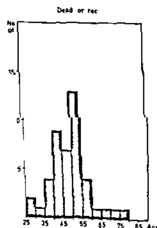


Fig 5 Distribution of age of patients who either died or got recurrence within five years

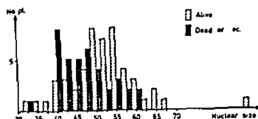


Fig 6 A diagram with the average size of nuclei in the individual patients with uterine cervical cancer

of factors among which the local spread of tumours is the most outstanding one

The here submitted study of the interrelation of clinical stage of lesions and 5 year survival of patients is analogous with series dealt with in the literature As regards stage I, 5 year survival rates are seen in 90 per cent of cases the percentage falling to 11 during stage IV (18)

In many studies of survival rates in cases of cervical cancer, the correlation between survival rate and histologically demonstrable degree of differentiation of tumours has been analysed For instance, *Atkin et al* (2, 3), *Miller et al* (24), *Gilmour et al* (9), and *Wentz et al* (38, 39) have all found the prognosis of highly differentiated tumours to be more favourable than that of the low differentiated ones

In the present study, as well as in a few of the previously published works within the same field (*Linell et al* (23) and *Brack et al* (6)), such relation between histological degree of differentiation and prognosis was not demonstrable It applies to most tumours that prognoses of poorly differentiated lesions

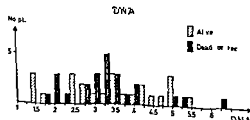
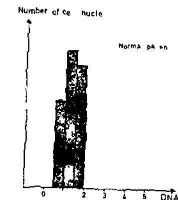
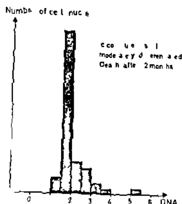


Fig 7 Diagram with the average DNA-content of nuclei in the individual patients with carcinoma of the uterine cervix





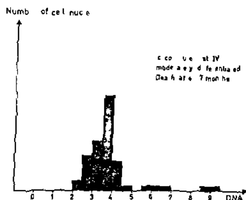
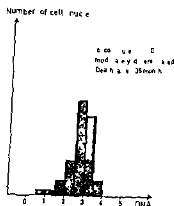
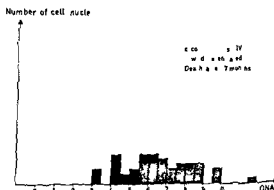


Fig 8 Examples of DNA karyograms of various tumours of the uterine cervix the DNA in arbitrary units

are unfavourable The present study can be taken as an expression of the fact that the histological structure of the cervical tumours fails to disclose much concerning the bio logical character of the tumour tissue It should be mentioned, however, that fluctuating degrees of differentiation may be encountered in one and the same malignant tumour No more than 3 or 4 slides from each patient have been examined in the present study

According to our studies of nuclear sizes differences between those in normal and malignant tissue were significant, but any differences within the individual clinical stages could not be demonstrated The average size of nuclei in invasively growing cervical tumours was almost twice the size of normal cell nuclei This is in accord with the results obtained by Reagan and coworkers in studies of nuclear sizes in non malignant and malignant portio-tissue (29) As early as in 1907





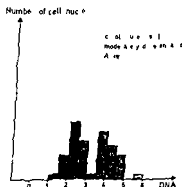


Fig 9 Examples of DNA karyograms of various tumours of the uterine cervix, the DNA in arbitrary units

Heiberg (12, 13) published his observation of a difference in nuclear sizes in normal and malignant materials. Later, the nuclear sizes in malignant, human tumours have been studied by *Strodtbeck* (24), *Cramer* (8), and *Oluf Petersen* (28), but these authors failed to take into consideration whether prognosis and nuclear size might be mutually interrelated. A comparison of the histological degree of differentiation and DNA content and/or nuclear size showed that DNA values of an order comparable with those found in invasively growing tumours were demonstrable as early as during the histologically verified premalignant stage: carcinoma in situ. *Brandau* (7) found modal DNA values to be within the "malignant" range in three patients out of seven with carcinoma in situ. A series of investigations (12, 32, 33, 37, 41) on the chromosomal content in tissue histologically diagnosed as carcioma in situ are available. Many of the results indicated that the chromosome karyograms might be identical with those observed in invasively growing tumour tissue.

The present study of nuclear sizes and DNA contents in carcinoma in situ supports the postulation that chromosomal changes represent an early symptom of malignancy. Average values were in all cases found to be elevated. Cells with diploid or near diploid content of DNA were demonstrable, however, in most tumours. Differences between nuclear sizes in malignant tumours and 5 year survival rates were not found to be significant. A certain tendency suggested that the average nuclear size in patients who either died or in whom tumours recurred might be inferior to that observed in surviving patients, but such tendency was not significantly different in the two groups.

As mentioned in the introduction, *Atkin and coworkers* (2) classified their series of patients with cervical cancer into two groups, one in which the DNA content and/or nuclear size were in the hyperdiploid region and another in which the DNA content was in the tetraploid range; these authors demonstrated that the prognosis was least favourable in patients with hyperdiploid content of DNA.

Our series of patients could not be divided into two such groups although we admittedly have found two modal values, one within the diploid region, the other in the tetraploid; the tetraploid modal peak may have been an expression of the premitotic G_2-component in a unimodal tumour (40).

In accordance with the described measurements of nuclei, the cellular nuclei of tumours were found to have a high DNA content; the level was highest during the clinical stage II. Furthermore, values were found to be widely scattered. Varying forms of ploidy were encountered in most cases, and most tumours in our series were either within the triploid or the aneuploid region. It seems rather surprising that Atkin observed a preponderance of diploid nuclei during the clinical stage I in which the prognosis otherwise is held to be fair (in the present study, 5 year survival rates were observed in 90 per cent of all cases), while hypotetraploid nuclei were found to predominate during stages III and IV.

Also *Grundmann and coworkers* (11) examined the content of DNA in cervical epithelium in normal tissue as well as in tissues during transition from precancerous stages to fully developed cancer. In the fully developed carcinoma, the DNA-content per nucleus was twice that in normal tissue although the scatter of values was much wider. The authors suggested that the scatter might be explicable by the fact that many cells in the proliferating tissue were about to enter into mitosis, i.e. that values in diploid nuclei were in the range between diploid and tetraploid regions.

Sandritter et al. (31) examined the DNA content in 30 different types of tumours in man but failed to demonstrate any correlation between clinical course and DNA content. In the case of malignant tumours Sandritter observed not merely an increase in DNA-content but also a wide scatter of values. *Leu and coworkers* (22) examined DNA contents and/or nuclear sizes in ma-

lignant tumours of the bladder but failed to demonstrate any correlation between the two parameters and prognosis, a correlation between DNA-content and nuclear size was not demonstrable either The latter authors (22) held that tumours of the bladder initially might be diploid, subsequently progressing first into the hypotetraploid region and finally into the aneuploid range *Levi et al* found a similar development of the chromosomal pattern in mice into which tumours of the bladder were induced by amino-acethyl fluorene (22)

The above hypothesis may apply also to cervical tumours and, if so, the stage at which biopsies are taken during progression of the tumour tissue may be decisive for the values to be obtained as regards DNA-content and nuclear size Furthermore, the polyploidy which is known to rise parallel with age (20) may also play a certain role

Inui and coworkers (14) did not observe any correlation between DNA-contents and degrees of malignancy in cases of human tumours involving the stomach *Jacobsen* (15) did not either observe a correlation between rates of survival and DNA-content in cases of tumours involving the prostate, nor did he find a relation between histologically demonstrable malignancy and DNA-content in the prostatic epithelium *Greisen's* studies (10) of the DNA-content in bronchogenic carcinomata did not disclose any differences in modal values in bronchogenic carcinomata, whether they were differentiated to high, moderate or low degrees, although the average content of DNA at the individual degrees of differentiation seemed to differ slightly Thus, according to most of the available studies of DNA-contents and nuclear sizes in tumours in man, malignant tumours have been found to have a higher content of DNA and larger sizes of nuclei than otherwise seen in non-malignant tissue, moreover malignant tumours have been found to present an aneuploid DNA-pattern, finally, a relation between DNA-content and/or nuclear size and degree of malignancy cannot be established

Malignant tissue from patients with tumours involving the cervix uteri has been found to contain increased volumes of DNA in the nuclei of tumour cells together with significantly enlarged cellular nuclei By way of comparison between DNA-content and nuclear sizes in the individual patients, these parameters were not found to be correlated probably owing to the wide scatter of values, or rather as a result of an actual variation within the individual tumour tissues, the explanation being that different biopsy material had been used for determinations of DNA content and measurements of nuclear sizes

On the basis of findings in our study of patients with cervical cancer it can be concluded that clinical stages and 5-year survival rates are correlated and also that the DNA-content and/or nuclear size in patients with cervical tumours were higher than contents and sizes in non-malignant tissues We have not been able to confirm the observation made by other authors according to which the DNA-content and/or nuclear size, the histological morphology, and the degree of malignancy expressed by 5-year survival rates were presumed to be correlated

Thus, the results obtained are in support of the hypothesis that certain fundamental cytological changes may have occurred in the tumour cells (increased DNA content and nuclear size), whereas behaviour of cells in the organism (the prognosis) is determined rather by factors other than these, for instance, by immunological factors.

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THE INTRARENAL VASCULAR ALTERATIONS IN RADIATION NEPHRITIS AND THEIR RELATIONSHIP TO THE DEVELOPMENT OF HYPERTENSION

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Unilateral radiation nephritis was induced in 20 rats This regularly resulted in the development of hypertension, but only after removal of the non irradiated kidney The increase in blood pressure was unrelated to uraemia The irradiated kidneys showed typical micro-angiographic alterations consisting in a reduction of the cortical vasculature, whereas the medullary vasculature was well visualized These alterations were ascribed to glomerular lesions induced by the irradiation Renal arteriolosclerosis was mild or absent The juxtaglomerular cell granulation was increased in the non irradiated kidney early after irradiation and tended to return to normal levels by prolongation of the interval from irradiation to removal of the non irradiated kidney It is suggested that (i) the non irradiated kidney exerts a protective action on the blood pressure level in unilateral radiation nephritis (ii) the development of hypertension after removal of the non irradiated kidney is due to an altered vascular pattern in the irradiated kidney resulting in a relative cortical ischaemia and (iii) the radiation causes an acute damage of the juxtaglomerular cells which in the present experiments was reversible

It is known since long that radiation therapy of the kidney area in man may lead to the development of arterial hypertension (*Luxton* 1953 *Wilson, Ledingham & Cohen* 1958) Recent data suggest that the blood pressure elevation is produced by an increased liberation of a pressor substance from the irradiated kidney presumably renin (*Schwartz, Alpert, Sommers & Mason* 1969), and it has been proposed that this liberation is a consequence of renal ischaemia resulting from radiation damage of the intrarenal arterioles (*Wachholz & Casarett* 1970) The latter workers noted, however, that the alterations in the walls of the intrarenal arterioles may be quite subtle, and others have shown that irradiation of the kidney may lead to hypertension in the absence of any histologically demonstrable lesions in the intrarenal arterioles (*Wilson et al* 1958, *Fisher & Hellstrom* 1968)

In a series of recent studies we have shown that various experimental kidney diseases will produce alterations in the distribution of the finer intrarenal vasculature which, unrelated to any renal arteriolosclerosis, may

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form a morphological basis for a relative cortical ischaemia and hence hypertension (*Ljungqvist* 1969, *Ljungqvist* & *Richardson* 1969, *Ljungvist, Sallstrom* & *Bibcrfeld* 1970) The present investigation was undertaken with the aim to see whether such alterations in the intrarenal vascular pattern would be produced also by renal irradiation

MATERIAL AND METHODS

Female Sprague Dawley rats were used for the experiments They were kept on tap water *ad libitum* and a laboratory chow containing 0 4 per cent sodium chloride throughout the experiments Before renal irradiation the blood pressure of the rats was assessed on three different occasions with the tail pletysmographic method and with the rats under light ether anaesthesia

The left kidney of the rats was irradiated with ^{60}Co γ rays and with the rats under intraperitoneal nembutal anaesthesia During the irradiation the rats were in prone position and observed on a television screen The opening of the tube diaphragm measured 3 × 4 cm whereas the field on the skin was restricted to 2 × 3 cm by the introduction of 7 cm thick lead plates between source and skin By this arrangement the penumbra was reduced The source skin distance was 40 cm and the organ dose was estimated at a depth of 2 cm to 90 per cent of the skin dose The accuracy of the dose was assessed by the use of a condensator chamber Preliminary experiments showed that a single organ dose of 2000 rad led to radiation nephritis with a low mortality rate and this was used in all experimental rats

The material consists of the 23 irradiated rats that survived the experimental procedures and of 10 control rats not exposed to irradiation The blood pressure and body weigths were measured once a week In all rats the initial blood pressure level was well below 140 mm Hg Hypertension was considered to have developed when the blood pressure became stabilized at or above 140 mm Hg provided that this included a rise from the original level of at least 15 per cent

All rats were still normotensive 10 weeks after irradiation Since there are data suggesting that the normal kidney exerts some protective action on the blood pressure level of rats with unilateral renal parenchymal disease (*Ljungqvist* & *Richardson* 1969) 8 rats were subjected to right nephrectomy during the 11th post irradiation week

The remaining 15 irradiatated rats were still normotensive during the 19th post irradiation week and 7 of these rats were subjected to right nephrectomy at that time Among the remaining 8 rats 5 were subjected to the left nephrectomy during the 28th post irradiation week when they were still normotensive, and the last 3 rats were nephrectomized during the 35th post irradiation week when still normotensive Control rats were nephrectomized at corresponding intervals after the start of the experiments

Twenty of the irradiated rats developed hypertension at various intervals following nephrectomy (Table 1) and three rats remained normotensive All hypertensive rats showed morphological evidences of radiation nephritis No such lesions were found in the three normotensive rats These rats were eliminated from the material and are thus not included in the Tables

The removed right kidneys of control as well as irradiated rats were subjected to arterial infusion of a 7 5 per cent aqueous suspension of fine grain barium sulphate (Micropaque) for combined stereomicro angiographic and histological examinations as previously described (*Ljungqvist* 1963)

At the end of the experiments the rats were anaesthetized the heart exposed and blood withdrawn by heart puncture for determination of serum creatinine Thereafter 5 ml of the Micropaque suspension was injected into the left atrium This led to the death of the animal within a few minutes after which a catheter was inserted into the aorta with the tip located just above the origin of the left renal artery This catheter was connected to a bottle containing Micropaque suspension which was infused over a period of 30 minutes and at a pressure successively increasing from 60 to 150 mm Hg The kidney was then removed and fixed in a modified Helly s solution for 24 hours

A second catheter was inserted into the aorta and directed towards the heart Micropaque was infused as above in order to fill the myocardial vessels for studies of the myocardial vascular pattern in hypertensive cardiac hypertrophy This will be the subject of a future report Before fixation of the heart in 10 per cent neutral formalin it was weighed for determination of the heart/body weight ratio

After fixation of the kidneys in a modified Helly s solution (*Ljungqvist* & *Richardson* 1969) they were processed for combined stereomicro angiographic and histological examinations The histological procedures included staining of one frontal section from each kidney by the Bowie method for visualization of the juxtaglomerular cell granules The degree of granulation of the kidney was expressed as the juxtaglomerular granulation index (JGI) which was based on the counting of all glomeruli in the section and the estimation of the degree of granulation of each juxtaglomerular apparatus encountered (*Hartroft* & *Hartroft* 1953) Other kidney sections were stained with haematoxylin-eosin Ladewig s stain for fibrinoid material and with van Gieson s connective

tissue stain counterstained with Weigert s elastin On examinations of these sections the degree of glomerular damage was appreciated by calculating the glome described the count and the glau s glomerulus from + (slight alteration) to + + + (severe alteration) The number of glomeruli denoted as +, + + and + + + were multiplied by factors 1, 2 and 3 respectively The sum of the products per 100 glomeruli constitutes the GLI of the kidney, the highest possible value thus being 300

RESULTS

Clinical findings In the present material unilateral radiation nephritis regularly led to hypertension after the non irradiated kidney had been removed (Table 1) Table 1 also shows that the latent period for hypertension to develop after removal of the non irradiated kidney tended to be shorter with prolongation of the interval from left renal irradiation to right nephrectomy The total period from renal irradiation to the development of hypertension was, however, shorter in the rats in which contralateral nephrectomy was performed after 11 and 19 weeks (Groups A and B) than in the rats in which nephrectomy was performed later (Groups C and D)

In an attempt to evaluate the significance of the duration of the blood pressure elevation for the evolution of the renal lesion 11 rats were killed after hypertensive periods covering between 1 and 3 weeks and 7 rats

TABLE 1 *The Effect on the Blood Pressure of Left Sided Renal Irradiation Followed by Right Sided Nephrectomy at Different Intervals Thereafter (Groups A D) A Control Group of Non Irradiated Rats Is Included (E)*

Group	Rats No	B p 1	Int 1	B p 2	Int 2	Int 3	B p 3
A	8	110	11	100	16	5	185
B	7	120	19	115	10	3	175
C	3	120	28	115	11	4	165
D	2	115	34	110	6	4	150
E	10	115	11 34	115	–	–	120

B p 1 Mean blood pressure in mm Hg at left sided kidney irradiation
Int 1 Interval in weeks between left sided kidney irradiation and right sided nephrectomy
B p 2 Mean blood pressure in mm Hg at right sided nephrectomy
Int 2 Interval in weeks from right sided nephrectomy to development of hypertension
Int 3 Interval in weeks from development of hypertension to death of animals
B p 3 Mean blood pressure in mm Hg at the time of death of animals

TABLE 2 *Heart/Body Weight Ratios (Ratio = Heart Weight × 1000/Body Weight) and Serum Creatinine Levels (Creat) at the Time of Death of Animals with Radiation Nephritis Induced Hypertension of Various Lengths (I III) A Control Group of Non Irradiated Rats Is Included (IV) The Figures Are Mean Values ± SE*

Group	Rats No	B p	Dur	Ratio	Creat (mg %)
I	11	175	1 3	5 8±0 3	1 0±0 2
II	7	170	4–6	6 0±0 2	1 2±0 3
III	2	195	12	7 2±0 3	0.8±0 1
IV	10	120		4 8±0 1	0 7±0 1

B p Mean blood pressure in mm Hg at the time of death of animals
Dur Duration of hypertension in weeks

TABLE 3 *Glomerular Lesion Index (GLI) and Juxtaglomerular Granulation Index (JGI) in Right (R) and Left (L) Kidneys Examined at Various Intervals After Left Sided Renal Irradiation (A D) A Control Group of Non Irradiated Rats Is Included (E) The Figures Are Mean Values ± SE*

Group	Rats No	Int	Dur	GLI L	GLI R	JGI R	JGI L
A	8	11	32	<1	28±9	35 8±2 2	10 7±1 4
B	7	19	32	<1	13±4	22 3±2 4	11 8±2 1
C	3	28	43	<1	16±9	23 0±3 6	10 6±2 6
D	2	34	44	<1	19±2	19 8±0 2	9 9±3 2
E	10	11–34	32–44	<1	<1	10 7±1 1	10 2±0 9

Int Interval in weeks between left sided kidney irradiation and right sided nephrectomy
Dur Duration (weeks) of experiment from left sided renal irradiation to death of animals

TABLE 4 *Glomerular Lesion Index (GLI) and Juxtaglomerular Granulation Index (JGI) in the Left Kidney of Rats with Respect to the Duration of the Hypertension Induced by Left Sided Renal Irradiation and Right Sided Nephrectomy (I–III) A Control Group of Non Irradiated Rats Is Included (IV) The Figures Are Mean Values ± SE*

Group	Rats No	Dur 1	Dur 2	B p	GLI	JGI
I	11	33± 1	1–3	175	17± 6	13 5±1 3
II	7	37± 3	4–6	170	15± 4	8 0±0 6
III	2	33± 1	12	195	35±11	7 6±0 7
IV	10	32—44	–	120	1	10 2±0 9

Dur 1 Duration (weeks) of experiment from left sided renal irradiation to death of animals
Dur 2 Duration of hypertension in weeks
B p Mean blood pressure in mm Hg at the time of death of animals

after hypertensive periods of 4–6 weeks, the two remaining rats were killed after 12 weeks of hypertension(Tables 2 and 4) All hypertensive rats showed cardiac hypertrophy to judge from the significant increased heart/body weight ratio, this increase was more pronounced the longer hypertension had been present (Table 2)

The serum creatinine levels of the hypertensive and control rats are shown in Table 2 It can be seen that there is some increase in the serum creatinine level in the hypertensive rats when compared with the control rats, but this increase is mainly due to a severe elevation in one rat in Group I (2 2 mg per cent) and one rat in Group II (2 5 mg per cent) If these two rats are disregarded, the mean serum creatinine level of the hypertensive rats is *0 8 ± 0 1 mg per cent* which is not statistically different from the level in the control rats ($p > 0\ 10$)

Morphological findings The gross and histological features of the kidney in radiation nephritis have been described in detail by previous workers and are well defined (*Rubin* & *Casarett* 1968) Basically these lesions include progressive contraction of the kidney due to glomerular sclerosis tubular atrophy with collapse or dilatation of the lumen and usually a severe degree of interstitial fibrosis and sclerosis In the present material only three of the rats showed this fully developed picture in the irradiated kidneys (Figs 1 and 2) These rats were killed at different intervals after renal irradiation and had been hypertensive for various lengths of time In the remaining 17 irradiated rats the histological kidney lesions were



Fig 1 Survey picture of renal cortex from a rat with radiation nephritis and hypertension There s interstitial fibrosis and irregular atrophy of the tubules The glomeruli show varying degrees of sclerosis van Gieson × 115

comparatively mild although obvious A conspicous feature was the paucity of contrast medium in the glomerular capillaries when compared with the kidneys of the control rats The glomerular tufts appeared collapsed, but histologically visible glomerular degeneration and sclerosis which forms the basis for the GLI determinations, was mild when compared with previous observations in renal hypertensive rats (*Ljungqvist* 1969, *Ljungqvist* & *Richardson* 1969 *Ljungqvist, Sallström* & *Biberfeld* 1970) Extraglomerular vascular alterations were sparse, and in many kidneys entirely absent (Figs 2 and 3) When present they consisted in focal hyalinosis or fibrinoid necrosis of the walls of finer arteries and arterioles, in some places associated with the formation of fibrin thrombi on the damaged intima Dilated tubules often contained casts and the increased amount of interstitial fibrous tissue displayed focal infiltration of lymphocytes The tubular and insterstitial alterations were both most marked in the cortical area. In most kidneys the lesions were distributed throughout the cortical area but in some instances they were restricted to the middle portion of the kidney, leaving the two poles unaffected

In Table 3 the degree of glomerular degeneration and sclerosis (GLI) and the degree of juxtaglomerular cell granulation (JGI) are presented with respect to the time interval from irradiation to death In Table 4 the same observations are presented with respect to the duration of the blood pressure elevation There is no significant difference

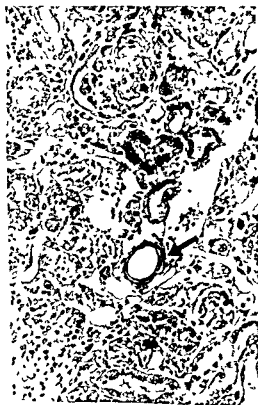


Fig 2 Section from renal cortex of a rat with radiation nephritis and hypertension The glomerulus included in the picture (*top*) shows severe sclerosis and its capillaries are almost completely occluded One cross sectioned cortical arteriole is seen (*arrow*) This contains contrast medium and its wall is unaltered van Gieson elastin × 190

between the GLI values of Groups A B, C and D in Table 3 (p > 0 1) Nor is there any difference in GLI between rats with hypertensive periods of less than 3 weeks and rats with 4–6 weeks of hypertension (Table 4), the GLI in the two rats in which hyper tension was allowed to persist for a consider able lenght of time (Group 3) is possibly higher, but these observations are too few to permit of any statistical analysis

In Table 3 the differences in JGI between the right and left kidneys are statistically significant for groups A B and C (p < 0 001, p < 0 01 and p < 0 05) but not for group E



Fig 3 Section from renal cortex of a rat with radiation nephritis and hypertension showing a glomerulus with minimal histological changes and well open capillary lumina The afferent arteriole contains contrast medium (*arrow*) but this has not penetrated into the capillary tuft A neigh bouring cortical arteriole is seen which contains contrast medium and shows a normal wall van Gieson elastin × 475



Fig 4 Micro angiogram from the non irradiated kidney of a hypertensive rat in which the contra lateral kidney had been irradiated The pattern is entirely normal with well visualized cortical and medullary vasculatures (c f Fig 5) × 12

(p > 0 05) for group D the observations are too few It can be seen that the difference in JGI between the right and left kidneys of the experimental animals is due to a signifi cant increase in JGI of the non irradiated (right) kidneys when compared with the kidneys of the control rats In Table 4 no significant differences in JGI between the experimental animals (Groups I–III) and the controls (Group IV) were recorded (p>0 05)

The micro angiograms of the right kidneys and of the kidneys of the control rats showed completely normal pictures with a good visualization of cortical as well as medullary vasculatures (Fig 4) In the irradiated kidneys the following alterations were noted in the survey micro angiograms a) an in complete visualization of the cortical vascula



I
as the medullary asculature is abundant In tl e cortex the arterie and arter oles d splay rre gulari es n the r courses and cal b es V sua ized glomerul are few The poor sual zat on of the postglomerular cap lary ne work s consp cuous (c f F g 4) × 12

ture whereas the visualization of the medullary vasculature was normal or improved (Fig 5) b) tl e visualization of some veins part cularly along the corticomedullary border and in the j ixtamedullary zone of tl e cortex c) spiralling of visualized cortical arteries and arterioles in the kidneys with the most pronounced histological lesions (Fig 6) On closer examination it was observed that the reduction of the cortical vasculature was due to a poor visualization of the glomerular tufts and postglomerular vessels Many cortical arterioles were seen to end blindly or in the form of a simplified glomerular tuft without visualization of a postglomerular vasculature (Fig 6) In the juxtamedullary zone many aglomerular arterioles were seen to descend into the medulla where they contributed to a well developed medullary vasculature (Fig 7)

When the micro angiograms were compared with the corresponding histological sections it was found that in the cortex many afferent arterioles ended as blind vessels at the vascular pole of unfilled glomeruli (Fig 3) some of which showed degeneration and sclerosis of varying severity (Fig 2) In the juxtamedullary zone incomplete glomerular filling did not interfere with the visualization of the postglomerular vessels and in many instances arterioles were seen to by pass the



Fig 6 Micro angiogram from tl e irrad ated k d ley of a rat w th rad ation nephrit s and hypertension showing poor v sual zat on of the cortical vascula tu e wh ch contains a number of meandering and p rall ng arteries and arterioles (*smaller arrows*) Many cort cal arterioles end bl ndly Veins are seen along the cort comedullary border (*larger arrows*) × 30



Fig 7 Micro angiogram from the irradiated kidney of a rat with radiation nephritis and hypertension In the cortex many arterioles end blindly and glomeruli and postglomerular capillaries are few A number of aglomerular arterioles (*arrow*) is seen to contribute to the well visualized medullary vasculature × 30

vascular pole of unfilled and sometimes degenerated or sclerosed glomeruli and descend into the medulla

The micro angiographic alterations were of a similar degree and extent in most kidneys but in three they were particularly pronounced These cases included those two in which hypertension had been present for a longer period of time (Group III Table 4) The third rat with a pronounced pathological intrarenal vasculature had not been hypertensive for a particularly long period (1 week) but in this kidney the GLI was high (34)

DISCUSSION

The present investigation has shown that irradiation of one kidney in situ can when leading to radiation nephritis also lead to hypertension It cannot be definitely assessed from the obtained data whether the development of hypertension requires the removal of the non irradiated kidney since it cannot be excluded that hypertension would have developed if the observation time had been extended beyond the 35th post irradiation week To judge from experiments performed by others, contralateral nephrectomy is not necessary (*Fisher & Hellstrom* 1968, *Schwartz et al* 1969) The results of the investigation indicate however, that the non irradiated kidney exerts a protective action on the blood pressure This is evidenced by the fact that removal of the non irradiated kidney 11–19 weeks after irradiation resulted in the development of hypertension around the 28th post irradiation week whereas the non nephrectomized rats were all normotensive at this time, removal of the non irradiated kidneys 28–35 weeks after irradiation resulted in hypertension around the 40th post irradiation week

The mechanism leading to elevation of the blood pressure in radiation nephritis is obscure The present study has shown that causes other than uraemia have to be considered Recent data strongly suggest that the blood pressure elevation is of true renal origin probably resulting from the liberation of a pressor substance into the circulation (*Schwartz et al* 1969) It is known that renin will be released from the juxtaglomerular cells of the cortex during renal ischaemia such as is produced by renal artery stenosis The blood pressure elevation complicating various parenchymal kidney diseases including radiation nephritis has been attributed to a reduction of the renal circulation by damage and thickening of the walls of the intrarenal arteries and arterioles (*Wachholz & Casarett* 1970) but others have shown that hypertension may develop in the absence of any such lesions (*Wilson et al* 1958 *Fisher & Hellstrom* 1968)

In previous investigations we have shown that experimentally induced renal parenchymal disease often leads to hypertension in

the absence of any alterations in the extraglomerular blood vessel walls (*Ljungqvist & Richardson* 1969, *Ljungqvist et al* 1970) The crucial finding in these investigations was that glomerular damage, which is part of the parenchymal diseases investigated, had produced an altered intrarenal microvascular pattern suggestive of ischaemia of the cortex with maintenance or even enhancement of the medullary circulation In the irradiated kidneys of animals comprised in the present material this microangiographic pattern of a relative cortical ischaemia was again produced although histologically evident glomerular degeneration and sclerosis were comparatively mild However, such glomerular lesions are known to be preceded by ultra structurally demonstrable obliterative changes in the glomerular capillaries (*Madrazo, Suzuki & Churg* 1969) and it is probable that these early stages of glomerular alterations were predominating in the present material

In a recent micro angiographic study of the rabbit kidney with radiation nephritis, vascular alterations similar to those reported herein were observed but the animals remained normotensive (*Scanlon* 1970) The failure of these animals to develop hypertension can be ascribed to the fact that unilateral renal irradiation was performed and the opposite kidney was not removed It is well known that the rabbit is less liable than the rat to develop hypertension in connection with unilateral renal disease

Scanlon (1970) like earlier *Maier & Casarett* (1964) also observed spiralling of the intrarenal arteries and arterioles This feature has previously been noted both in normotensive and hypertensive states and can be ascribed to contraction of the kidney parenchyma (*Ljungqvist* 1963)

It is generally accepted that the degree of juxtaglomerular cell granulation in the rat is a measure of the renin content of the cells, and in certain instances also of the rate of liberation of renin It has been pointed out, however that in theory, an increased degree of granulation may alternatively reflect a decreased liberation of renin from the cells at a maintained production of the substance (*Ljungqvist* 1969) Consequently, a decreased degree of granulation may either represent a decreased production and liberation of renin or an increased liberation leading to depletion It would thus seem plausible that an equilibrium between the rate of production and liberation of renin may be established at different degrees of juxtaglomerular cell granulation, depending upon the type of stimulus acting on the cells The absence of any significant change of JGI in the irradiated kidney of most of the rats does therefore not contradict the possibility of a renin induced elevation of the blood pressure

The cause of the elevation of the JGI in the non irradiated kidneys of animals comprised in the present material is obscure (Table 3 Groups A, B, C and D) In an attempt to explain this feature it should be recalled that all these kidneys were removed from normotensive animals and at different intervals following irradiation of the opposite kidney It is reasonable to assume that the high single irradiation dose to the left kidney caused an acute functional disturbance of various cell functions in this kidney, including the function of the juxtaglomerular cells This might well be expected to result in a compensatory reaction of the juxta glomerular cells in the opposite kidney, morphologically reflected as an increase in their degree of granulation This explanation is in some measure supported by the previous demonstration of an elevation of the JGI in the remaining kidney of unilaterally nephrectomized rats (*Ljungqvist & Wågermark* 1968) The return towards more normal JGI levels in the right kidney by increasing post irradiation intervals (Table 3 Groups B C and D) can be explained by a fading of the acute irradiation damage of the juxtaglomerular cells in the left kidney

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HEPATIC CHANGES IN DOGS AND RATS INDUCED BY XYLIDINE ISOMERS

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The effect of the xylidine isomers 2,4-, 2,5-, and 2,6-xylidine on the liver and kidneys of dogs and rats was studied The dogs received oral doses of 2, 10, and 50 mg/kg and the rats 20 100 and 500–700 mg/kg daily for 4 weeks The xylidine isomers affected the liver but produced *different hepatic changes in the dog and the rat.* In the dog all 3 isomers induced a fatty degeneration of the liver In the rat the 3 isomers caused a hepatomegaly mostly with a normal appearance of the liver cells The enlargement of the liver was greatest for the 2,4 isomer and least for the 2,6 isomer No renal lesions were found in any of the dogs or rats at any dose level

The knowledge of the toxicity of xylidine is based upon a variety of investigations Because of its hepatotoxic property xylidine was used in a study by *Szirbely et al* (1946) to evaluate various liver function tests in the dog Important information on the toxicity of xylidine has been obtained from investigations of food colours *e g* Ponceau 3 R, which are degraded in the body to different xylidines After oral administration of Ponceau 3 R high mortality growth inhibition, increased liver and kidney weights and liver tumours have been found in rats by *Grice et al* (1961) in rats dogs and mice by *Hansen et al* (1963) and in rats by *Mannel* (1964)

The xylidine isomers have a low acute toxicity (*Lindstrom et al* 1963) but show toxicity after repeated administration In rats the xylidine isomers differ from each other with regard to toxicity Hepatotoxicity was observed in rats fed 2,4 xylidine at different levels for a period of 3 to 6 months No similar effect was seen after feeding the same amount of 2,6 xylidine (*Lindstrom et al* 1963)

During subacute toxicity studies of xylidine containing compounds in rats and dogs it was noticed that dogs were more sensitive than rats and that there existed a difference in toxic effects in the various substances tested In the present study the effects of 2,4-, 2,5-, and 2,6-xylidine were evaluated in dogs and rats after daily administration for 4 weeks

METHODS

Dogs

Twenty 10–12 months old male and female beagle dogs weighing between 8 3–14 0 kg, were used as experimental subjects The animals were divided into 10 groups each consisting of 1 male and 1 female The animals were given food and water *ad libitum* The dogs were treated once daily for 4 weeks with 0 (control animals), 2 10 or 50 mg/kg of either 2,4-, 2 5- or 2 6- xylidine The compounds were given orally in capsules

Received 6 iv 71

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During the experiment the dogs were observed daily for clinical effects of the compound. Before the start and once a week during the experiment haematological and blood chemistry examinations were performed with standard methods. The following parameters were studied: haematocrit, haemoglobin concentration, red and white blood cell counts, differential leucocyte count, serum concentrations of glucose (GLOX® KABI AB, Stockholm, Sweden), bilirubin (Beckman Technical Bulletin No 6076 D), urea nitrogen (Hyland UN test, Hyland Laboratories, Los Angeles, California, USA), and total protein (*Weichselbaum* 1946). At the same intervals, the rate of clearance of sulphobromophthalein (BSP) was determined by measuring the dye retention 30 minutes after administration of 5 mg per kg body weight (*Cornelius* 1963). The dogs were sacrificed at the end of the experiment and subjected to complete autopsy. The liver and kidneys were examined microscopically.

Rats

One hundred approximately 2 month old male and female rats of the Sprague Dawley strain were used as experimental subjects. At start the males weighed between 272–310 g and the females 210–234 g. The animals were given commercial food and water *ad libitum*. The rats were divided into 10 groups each consisting of 5 males and 5 females. Initially the rats were treated orally by stomach tube once daily for 4 weeks with 0 (control animals), 20, 100 or 500 mg/kg of either 2,4-, 2,5- or 2,6-xylidine. After 2 weeks of treatment the dose administered to the high dose group was increased to 700 mg/kg. During the experiment the rats were observed daily for clinical effects of the compound. At the termination of the experiment haematological and blood chemistry examinations were performed with standard methods. The following parameters were studied: haematocrit, haemoglobin concentration, serum concentrations of urea nitrogen and ornithine carbamyltransferase (*Reichard* 1964). At the end of the experiment the rats were sacrified and autopsies were performed on all rats including those which died during the experiment. The livers and kidneys were examined microscopically.

Histopathology

Samples of the livers and kidneys were fixed in 10 per cent formalin and Bouin's fluid. Paraffin embedded sections were routinely stained with haematoxylin and eosin and van Gieson. Some sections were also stained with periodic acid Schiff's reagent (PAS), Weigert's stain for fibrin and Mallory's phloxine method (*Lillie* 1965). Frozen sections were stained with Sudan III for examination of fat. A few frozen sections were stained with lipase lead sulphide method for triglycerides (*Adams et al.* 1966).

RESULTS

Dogs

Clinical signs. The dogs of the 2 mg/kg groups were free of clinical signs. Dogs treated daily with 10 and 50 mg/kg of 2,4-, 2,5-, or 2,6-xylidine, showed vomiting which occurred from 0.5–4 hrs after treatment with all 3 substances. The vomiting was most frequent at the highest dose levels and occurred most often in dogs treated with 2,4-xylidine and least often in dogs treated with 2,6-xylidine. The 2 dogs receiving the highest dose of each compound also displayed a poor general condition and the body weights of these animals decreased between 1.6 and 3.8 kg during the experiment.

Laboratory investigation. Increased BSP retention was observed for all 3 isomers at the highest dose levels and in the female dog given 10 mg/kg of 2,6-xylidine (Fig. 1). At termination hyperbilirubinaemia (1.6–6.7 mg/100 ml) and hypoproteinaemia (3.9–4.2 g/100 ml) were observed in 1 of the 2 dogs which received 50 mg/kg of 2,5-xylidine and in both dogs which received 50 mg/kg of 2,6-xylidine (Table 1). The values for glucose, urea nitrogen, haemoglobin, haematocrit, red and white blood cells and differential counts of leucocytes were always within the normal ranges.

Gross pathology. At the high dose level of all 3 isomers the livers were slightly enlarged (Table 2). The dogs treated with 50 mg/kg of 2,5- and 2,6-isomers had fatty livers. The livers were yellow in colour, had a soft consistency, and a greasy cut surface. The bile was dark and thick. With the exception of the female dog treated with the 2,5-isomer the tissues of the dogs were icteric. In the dogs treated with 10 mg/kg of 2,5- and 2,6-isomers and 50 mg/kg of 2,4-isomer the livers were somewhat pale. The kidneys had a normal appearance in all dogs.

Microscopic pathology. In sections with slight to moderate fatty change small fat droplets were distributed in the periphero-

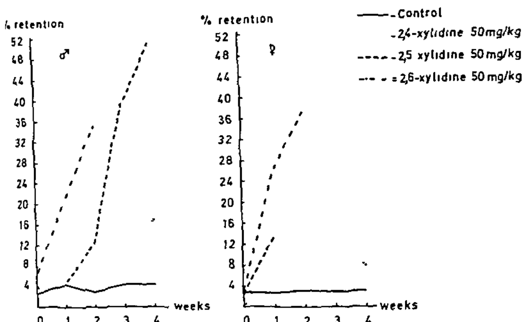


Fig 1 BSP retention (%) in the serum of male and female dogs after administration of xylidine isomers for 2–4 weeks Each point is an individual value

TABLE 1 *Summary of the Toxicity of Xylidine Isomers in Dogs*

Dog no	Treatment	Daily oral dose (mg/kg)	Sacrificed after number of days	Status of the liver*
1	Control	0	31	0
2			31	0
3	2 4 xylidine	2	31	0
4			32	0
5	2 4 xylidine	10	32	0
6			32	0
7	2 4 xylidine	50	19	+
8			22	+
9	2 5 xylidine	2	32	0
10			32	0
11	2 5 xylidine	10	29	+ +
12			29	0
13°	2 5 xylidine	50	28	+ + +
14			15	+
15	2 6-xylidine	2	29	+
16			29	+
17	2 6 xylidine	10	28	+ +
18			28	+ +
19°	2 6 xylidine	50	14	+ + +
20°			14	+ + +

* 0 No changes
\+ Slight fatty degeneration
\+ + Moderate fatty degeneration
\+ + + = Severe fatty degeneration.
° The dog showed a hypoproteinaemia a hyperbilirubinaemia and a general icterus.

TABLE 2 *Liver Weights (Expressed in Grams and As per cent of the Body Weight) in Male ar Female Dogs after Administration of Xylidine Isomers for 2–4 Weeks*

Dog no	Sex	Treatment	Daily oral dose (mg/kg)	Liver weight in grams	Liver weight in % of body weight
1	♂	Control	0	356	2 68
2	♀			459	3 17
3	♂	2,4 xylidine	2	330	2 17
4	♀			264	2 81
5	♂	2,4 xylidine	10	322	3 46
6	♀			284	3 55
7	♂	2,4 xylidine	50	328	4 21
8	♀			277	4 69
9	♂	2,5-xylidine	2	316	3 07
10	♀			377	3 37
11	♂	2,5 xylidine	10	278	2 60
12	♀			231	3 12
13	♂	2,5 xylidine	50	405	5 19
14	♀			324	4 63
15	♂	2,6 xylidine	2	315	2 74
16	♀			338	2 94
17	♂	2,6 xylidine	10	320	3 02
18	♀			263	3 55
19	♂	2,6 xylidine	50	360	4 29
20	♀			264	3 94

lobular cells (Fig 2) In severe lesions, a mixture of large and small fat droplets were diffusely distributed in the lobules

Using lipase-lead sulphide method for triglycerides the fat droplets were intensively stained brown to black In embedded sections of severely damaged livers the cells appeared empty due to the great number of fat droplets (Fig 3) Some isolated liver cells were necrotic or exhibited necrobiotic nuclear changes Bile thrombi and bile pigments were also observed when the hepatic lesions were severe

Fatty degeneration of the liver was observed at the high dose level of 2,4-isomer, at the medium and high dose levels of 2,5-isomer, and at all dose levels of 2,6-isomer The fatty degeneration was considered as slight at the high dose level of 2,4-isomer at the high dose level of 2,5-isomer with regard to the female dog, and at the low dose level of 2,6-isomer, as moderate at the medium dose level of 2,5-isomer with regard to the male dog and at the medium dose level of 2,6-isomer, and as severe at the high dose level of 2,5-xylidine with regard to the male dog and at the high dose level of 2 6 isomer (Table 1)

The kidneys had a normal appearance in all dogs

Rats

Clinical signs When rats were treated daily with 0 (control animals), 20, 100 and 500–700 mg/kg of 2,4-, 2,5- and 2 6 xylidine a decreased weight gain was observed at the high dose level in the male rats for all 3 isomers and in the female rats given 2,4 xylidine Nine mortalities occurred at the high dose level, 6 died after 2,4-xylidine (days 6–25), 1 after 2,5-xylidine (day 25), and 2 after 2,6-xylidine treatment (days 4–5)

Laboratory investigation At the high dose levels the haemoglobin concentration was decreased with all 3 isomers (Table 3) The haematocrit also showed a decrease though less pronounced than the haemoglobin (Table

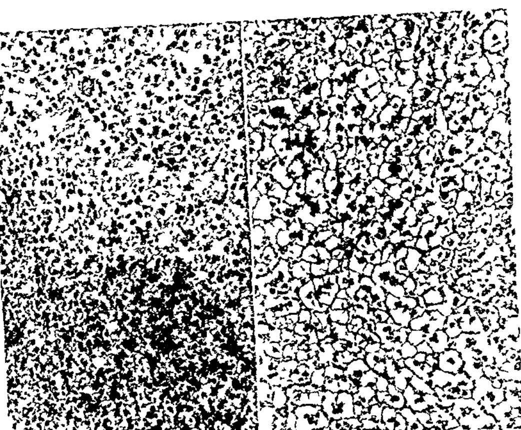


Fig 2 Fatty degeneration in the liver of a male dog given 10 mg/kg of 2 6 xylidine for 4 weeks The fatty change has a peripherolobular distribution (the lower area) At the top is a central vein Haemalum Sudan III 250 ×

Fig 3 Fatty degeneration in the liver of a male dog given 50 mg/kg of 2 5 xylidine for 4 weeks. The liver cells appear empty and clear due to fat droplets The bile canaliculi contain bile thrombi H & E 250 ×

3) In 13 rats increased values for OCT (53–455 Reichard units/ml) were demonstrated Seven of these rats were given 2 4 xylidine, 5 received 2 5 xylidine and 1 received 2 6-xylidine At the high dose level the OCT values were increased in 4 rats treated with 2 4 xylidine and in 5 rats treated with 2 5 xylidine At the medium dose level 1 rat receiving 2 4 xylidine showed an increase of OCT At the low dose level 2 rats given 2 4 xylidine and 1 rat given 2 6 xylidine had increased OCT values All values for urea nitrogen were within the normal ranges

Gross pathology The livers of the treated rats were enlarged (Table 4) The enlargement was most severe and observed at all dose levels in rats given 2 4 xylidine In rats treated with the 2 other isomers the enlargement was observed only at the high dose level In rats treated with 500–700 mg/kg of 2,4 xylidine the livers contained occasional reddish and greyish foci of a size of 0 5–2 mm. These foci were most evident in the rats which died Otherwise the livers did not show any change The kidneys had a normal appearance in all rats

Microscopic pathology At the high dose of 2,4 xylidine necrosis and vacuolization of the hepatocytes were observed in the livers of all rats (Table 5) The necrosis appeared as scattered foci (Fig 4), chiefly in the mid zone, of some of the hepatic lobules The

TABLE 3. *Haemoglobin Concentration and Haematocrit in Male and Female Rats after Administration of Xylidine Isomers for 4 Weeks*

Treatment	Dose (mg/kg)	Sex	No of animals	Hct (%) Mean	Hct (%) Range	Hgb (g/100 ml) Mean	Hgb (g/100 ml) Range
Control	0	♂	5	48	43-51	16 2	15 1-16 9
		♀	5	45	38-48	14 7	13 2-15 4
2,4-xylidine	20	♂	5	47	44-52	16 0	15 2-17 4
		♀	5	44	40-47	14 5	13 4-15 6
	100	♂	5	48	47-48	15 8	15 6-16 0
		♀	5	44	42-44	14 4	13 7-15 2
	500-700	♂	1	41		13 8	–
		♀	3	43	43-43	13 5	13 3-13 8
2,5-xylidine	20	♂	5	49	47-50	16 1	15 3-16 6
		♀	5	46	45-47	15 1	14 8-15 5
	100	♂	5	50	47-54	16 3	15 4-17 6
		♀	5	45	43-47	14 9	14 1-15 5
	500-700	♂	5	44	43-47	13 8	13 2-15 0
		♀	4	40	38-43	12 9	11 9-14 1
2,6-xylidine	20	♂	5	49	48 52	16 2	15 7-17 0
		♀	5	47	47-48	15 5	15 0-16 3
	100	♂	5	49	48-51	16 0	15 9-16 4
		♀	5	47	46-48	15 2	14 9-15 5
	500-700	♂	4	46	42-50	14 2	13 4-15 3
		♀	4	37	33-38	11 3	10 5-11 8

ABLE 4 *Liver Weights (Expressed in Grams and As per cent of the Body Weight) in Male and Female Rats after Administration of Xylidine Isomers for 4 Weeks*

p	Sex	No of animals	Treatment	Daily oral dose (mg/kg)	Liver weight in grams Mean	Liver weight in grams Range	Liver weight in % Mean	Liver weight in % Range
	♂	5	Control	0	12 5	10 9 13 8	3 61	3 16-3 81
	♀	5			8 2	7 4- 8 9	3 60	3 22-4 05
	♂	4	2,4-xylidine	20	15 3	13 9 17 0	4 37	4 16-4 68
	♀	5			10 7	10 0 11 3	4 61	4 24-5 10
	♂	5	2,4-xylidine	100	17 2	15 4-19 3	5 09	4 53-5 40
	♀	4			13 4	11 9 14 9	5 57	5 23 6 09
	♂	1	2,4-xylidine	500-700	23 8	23 8	8 31	8 31
	♀	3			20 2	17 2-22 5	8 12	6 83-9 13
	♂	4	2,5-xylidine	20	13 6	12 2-14 7	3 68	3 44 3 98
	♀	5			7 9	7 3- 8 6	3 33	3 07-3 67
	♂	5	2,5-xylidine	100	13 5	11 4-15 8	3 72	3 33-4 06
	♀	5			8 5	8 1- 9 0	3 45	3 11 3 76
	♂	5	2,5-xylidine	500-700	18 3	15 9-20 6	5 52	4 72 5 99
	♀	4			12 8	12 1 14 3	5 11	4 90 5 61
	♂	5	2,6-xylidine	20	12 9	11 2 14 6	3 68	3 39-4 04
	♀	5			8 8	8 0-10 0	3 56	3 32 3 87
	♂	5	2,6 xylidine	100	14 0	12 8-14 8	4 10	3 84 4 34
	♀	5			9 3	8 9- 9 6	3 86	3 66 4 02
	♂	4	2,6-xylidine	500-700	15 4	13 6-18 3	5 05	4 38 5 67
	♀	4			10 5	9 6-11 5	4 32	4 03-4 58

TABLE 5 *Summary of the Toxicity of Xylidine Isomers in Rats*

Group no	Treatment	Daily oral dose (mg/kg)	Status of the liver*
1	Control	0	0
2	2,4 xylidine	20	0
3	2 4 xylidine	100	0
4	2 4 xylidine	500–700 •	1
5	2 5 xylidine	20	0
6	2 5 xylidine	100	0
7	2 5 xylidine	500 700 •	2
8	2 6 xylidine	20	0
9	2 6 xylidine	100	0
10	2 6 xylidine	500–700 •	3

* 0 = Normal histological appearance
1 = Focal necrosis and vacuolization of the liver cells in all rats
2 = Fatty change in 1 female rat.
3 = Focal necrosis in 1 male and 1 female rat.
• The dose was increased to 700 mg/kg after 2 weeks

necrotic foci were generally small and well defined, the larger foci were more irregular in shape There was fresh hemorrhage within some necrotic areas and a cellular infiltration of mainly histiocytes and to a small extent neutrophilic granulocytes No fatty change was found In the centrolobular areas various sized vacuoles were common in the hepatic cells (Fig 5) The vacuoles appeared empty or had a somewhat filamentous content. The content of the vacuoles stained palely with eosin was not sudanophilic and gave a negative result with PAS Weigert fibrin stain, and Mallory s phloxine A slight proliferation of bile ducts was demonstrated at the high dose level of 2 4 xylidine

Fine fat droplets were observed in the peripherolobular areas in the liver of 1 female rat treated with 2 5 xylidine In rats given 2 6-xylidine necrotic foci were observed in 1 male and 1 female rat (Table 5) They had the same appearance as those in rats given 2 4 xylidine

The kidneys had a normal appearance in all rats

DISCUSSION

The results of this investigation show that xylidines affect the liver both in dogs and rats Increased values of BSP retention, bilirubin, and OCT were indicative of liver damage However, the morphological appearance of the livers was different in dogs and rats In the dogs all the 3 xylidine isomers induced a fatty degeneration of the liver In the rats the 3 isomers produced a hepatomegaly which was most evident for the 2,4-xylidine and least evident for the 2,6-xylidine The focal necroses and vacuoles of the hepatocytes which were observed at the high dose level of the 2,4 xylidine, may be related to the administered compound

It is well known that fatty change is a very common response of the liver to injury In spite of many different aetiologic factors the lipid in the liver cells is predominantly triglycerides Observations in the present study also indicated triglycerides in the liver cells The uniformity of response probably suggests that the pathogenesis of any fatty liver is related to one or few basic general mechanisms (*Lombardi* 1966) Triglycerides are probably secreted from the liver cells into the plasma as lipoproteins Inhibition of protein synthesis due to effects on the endoplasmic reticulum can cause an accumulation of triglycerides in the hepatic cell as a result of the failure of lipoproteins to be formed and

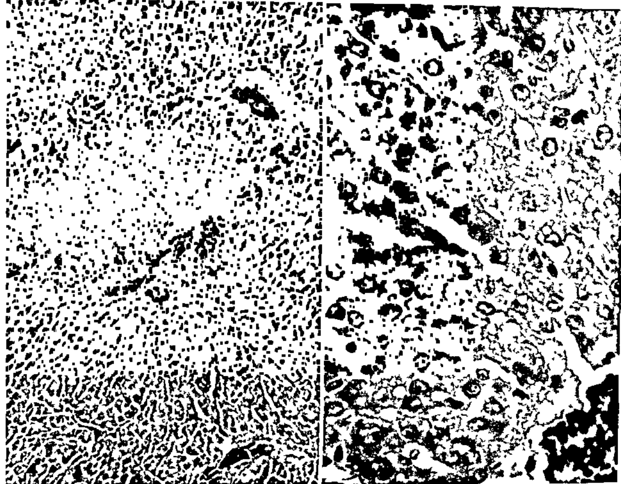


Fig 4 Focal necrosis in the liver of a male rat given 500–700 mg/kg of 2,4-xylidine H & E 150 ×
Fig 5 Vacuolization of the liver cells in a male rat given 500–700 mg/ of 2,4-xylidine Bottom right is a central vein H & E 650 ×

secreted (*Judah* 1969) Such a basic pathogenetic mechanism is believed to underlie the causation of fatty change induced in the liver of the rat by *e g* CCl_4 and phosphorus (*Lombardi* 1966). In the present investigation the fatty degeneration of the liver cells seen in dogs resembled that of phosphorus

The focal hepatic necroses in the rats probably do not represent a direct toxic effect Focal necroses, as observed in this study, are considered to be due to an insufficient nutrition of the liver cells on account of a low blood pressure and reduced circulation (*Himsworth* 1948, *Magnusson* 1963) Similar conditions may have occurred in the present investigation. This is suggested by the fact that the hepatic necroses were most numerous in the rats which died and which very likely had suffered from an insufficient blood circulation

The present investigation indicates that all 3 xylidine isomers induce a liver enlargement both in dogs and rats The findings in rats coincides with those obtained by *Lindstrom et al* (1963) showing that 2,4-xylidine causes a much more severe liver enlargement than 2,6-xylidine The enlargement of the liver in the dogs is probably explained by the fatty degeneration of the liver cells In the rats, the cause of the enlargement is not quite clear Mostly the liver cells had a normal histological appearance in the rats Cytoplasmic vacuoles of the hepatocytes were, however, observed in rats treated with the high dose of the 2,4-isomer These alterations are probably part of the explanation Another

one may be a proliferation of the smooth endoplasmic reticulum by the xylidine compounds It is known that chemical agents, *e g* barbiturates can induce a proliferation of the endoplasmic reticulum (*Remmer* 1964) In order to clarify this phenomenon completely, further investigations including electron microscopy are necessary

Though not reported here, a hyperkeratosis of the forestomachs was demonstrated in the rats at the high dose levels for all three isomers. This observation is in agreement with the investigation by *Lindstrom et al* (1963) The hyperkeratosis should be interpreted as expressions of an irritation of the stomach In the dogs, no signs of irritation to the stomach were found From other experiments it is, however, known that xylidines can irritate the stomach in dogs (own observations) Repeated administration of 2 6 xylidine in a dose of 100 mg/kg induced deep ulcers in the stomach of the dogs

Contrary to an earlier investigation (*Lindstrom et al* 1963) the [illegible]

[illegible] different duration of treatment employed *Lindstrom et al* (1963) treated animals for 3–6 months whereas in the present experiments animals were treated for only 2–4 weeks

At termination of the experiment a hypoproteinaemia was observed on a few occasions in the dogs This hypoproteinaemia is probably due to the liver damage as the liver synthesizes most serum proteins

It is evident that the toxicity of xylidines show great differences with regard to animal species and type of isomer As indicated by this study xylidines are more toxic for dogs than for rats Dogs seem to be about 10 times more susceptible than rats Concerning the isomers the dog and the rat also show a different pattern The present investigation shows that in rats 2 4 xylidine is more toxic than 2 6 xylidine *Lindstrom et al* (1963) also reported the same relationships In dogs the opposite seems to occur More dogs given 2 6-xylidine had to be killed before the planned termination and they suffered from the most severe liver damage (Table 1) This result suggests that in dogs 2,6 xylidine is more toxic than 2,4 xylidine

The differences in toxic effects produced by the xylidine isomers constitute interesting examples of structure activity relationships However, in order to elucidate the cause of the observed differences in toxicity, information of the metabolism of the different isomers in the 2 species are needed It would next be of interest to identify the toxic molecule, whether it is the xylidine or a metabolite, and to establish the time-course of its concentration in the liver

The toxicity of xylidine may be of significance to public health Man may be exposed to xylidine or xylidine yielding compounds at least in 2 ways, namely in food additives and in drugs The toxicity of xylidine has also been studied from these two points of view According to the present study the toxicity of xylidine displays species differences For that reason it may be very difficult to postulate the reaction of man when exposed to xylidine for a longer time

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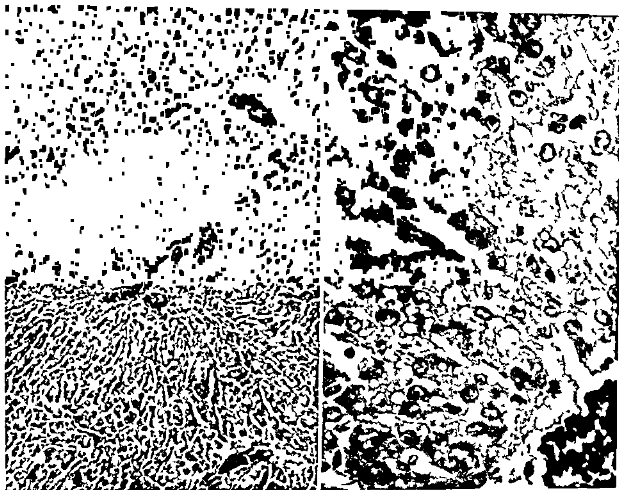


Fig 4 Focal necrosis in the liver of a male rat given 500–700 mg/kg of 2,4 xylidine H & E 150 ×
Fig 5 Vacuolization of the liver cells in a male rat given 500–700 mg/ of 2 4 xylidine Bottom right is a central vein H & E 650 ×

secreted (*Judah* 1969) Such a basic pathogenetic mechanism is believed to underlie the causation of fatty change induced in the liver of the rat by *e g* CCl_4 and phosphorus (*Lombardi* 1966) In the present investigation the fatty degeneration of the liver cells seen in dogs resembled that of phosphorus

The focal hepatic necroses in the rats probably do not represent a direct toxic effect Focal necroses, as observed in this study, are considered to be due to an insufficient nutrition of the liver cells on account of a low blood pressure and reduced circulation (*Himsworth* 1948, *Magnusson* 1963) Similar conditions may have occurred in the present investigation This is suggested by the fact that the hepatic necroses were most numerous in the rats which died and which very likely had suffered from an insufficient blood circulation

The present investigation indicates that all 3 xylidine isomers induce a liver enlargement both in dogs and rats The findings in rats coincides with those obtained by *Lindstrom et al* (1963) showing that 2 4 xylidine causes a much more severe liver enlargement than 2,6 xylidine The enlargement of the liver in the dogs is probably explained by the fatty degeneration of the liver cells In the rats, the cause of the enlargement is not quite clear Mostly the liver cells had a normal histological appearance in the rats Cytoplasmic vacuoles of the hepatocytes were however, observed in rats treated with the high dose of the 2,4 isomer These alterations are probably part of the explanation Another

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HISTOCHEMICAL STUDY OF MALLORY BODIES

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On a consecutive series of 34 liver biopsies a histochemical study of Mallory bodies has been performed. This has shown that the Mallory bodies contain unconjugated basic protein, ribonucleic acid, glycogen and some other non-acid carbohydrate component as well as phospholipid. Further NADH-diaphorase, acid phosphatase and esterase activity has been demonstrated. It is concluded that Mallory bodies thus contain more or less decomposed mitochondria, lysosomes, ribosomes and endoplasmic reticulum.

Since *F. B. Mallory* (1911) described a peculiar cytoplasmic hyaline degeneration in the liver cells of alcoholics with cirrhosis and named it "alcoholic hyaline", a number of histological procedures designed for the demonstration of these Mallory bodies have been presented (*Lacquer* 1950, *Roque* 1953). However, only a few histochemical studies of these structures have been made and most often on autopsy material (*Norkin et al.* 1960, *Becker* 1961).

The studies to be presented here attempt to investigate the histochemical composition of Mallory bodies and to contribute towards the elucidation of their pathogenesis on the basis of a consecutive series of liver biopsies with Mallory bodies.

MATERIAL AND METHODS

The material consists of 34 percutaneous liver biopsies all with typical Mallory bodies. The biopsies were selected from a material of 1,100 consecutive liver biopsies received at the pathological anatomical department, Kommunehospitalet, Copenhagen in the period 1/10 1965 – 4/10 1967 from six medical departments (*Copenhagen Study Group for Liver Diseases* 1969). In these Mallory bodies were demonstrated in a total of 62 (*Christoffersen* 1970). In 34 of these 62 cases, the number of Mallory bodies in each section was so high and the amount of tissue remaining in the paraffin block after routine histological sections had been cut sufficient to warrant a histochemical study of the Mallory bodies to be carried out. Five of the biopsies were performed by the *Vim Silvermann* method and twenty nine by the *Menghini* method. A control group of 102 histologically normal liver biopsies has been selected from the same 1,100 biopsies mentioned above.

The liver biopsies were 1–1.4 mm thick and 1.5–4.5 cm long. They were all fixed in buffered 3.6 per cent formaldehyde solution, dehydrated and imbedded in paraffin in the usual manner. After sections for routine histology had been cut, serial sections for histochemical tests for nucleic acids, proteins and carbohydrates were cut at 7 μm on a rotary microtome. Approximately every third section was stained routinely with haematoxylin and eosin to facilitate the identification of Mallory bodies.

In seven cases it has been possible to supplement the investigation with tests for lipids and enzymes as a part of the biopsy fixed in Baker's formol calcium at 4° C for 18 hours was at hand. The material was then transferred to 0.88 M gum acacia sucrose. Serial sections were cut at 7 μm on a SLEE cryostat.

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TABLE 3 *Histochemical Reactions Performed for the Demonstration of Carbohydrates*

Reaction	Macromolecule demonstrated	Chemically reactive group	Colour of final reaction
Periodic acid/Schiff (PAS) (McManus, J F A 1946)	glycogen, neutral mucopolysaccharides, glycoproteins, neutral mucoproteins, glycolipids, unsaturated polymerized lipids	1 2 glycol or certain derivatives of this group	magenta
Periodic acid/Schiff (PAS) following diastase digestion (Lillie, R D 1954)	neutral mucopolysaccharides, glycoproteins, neutral mucoproteins, glycolipids, unsaturated polymerized lipids	1 2 glycol or certain derivatives of this group	magenta
Aldehyde fuchsin pH 1 *(Spicer, Horn & Leppi)*	acid mucopolysaccharides, acid mucoproteins	polyanions *(sulphate)*	magenta
Alcian blue pH 1 *(Steedman, H F 1950)*	acid mucopolysaccharides, acid mucoproteins	polyanions *(sulphate)*	blue
Alcian blue pH 2 5	acid mucopolysaccharides, acid mucoproteins, nucleic acids, phospholipids	polyanions (sulphate, phosphate)	blue

TABLE 4 *Histochemical Reactions Performed for the Demonstration of Lipids*

Reaction	Macromolecule demonstrated	Chemically reactive group	Colour of final reaction product
Oil red O (French 1926)	hydrophobic unsaturated lipids	unsaturated free or ester bound long chain fatty acids	red
Sudan black B (Lison 1934)	hydrophobic unsaturated lipids, hydrophilic unsaturated lipids	unsaturated free or ester bound long chain fatty acids	black
Osmium tetroxide α naphthylamine (OTAN) (Adams 1959)	cholesterol esters, triglyceride esters	unsaturated fatty acid chains in hydrophobic lipids	black
	phospholipids	unsaturated fatty acid chains in hydrophilic lipids	red
Osmium tetroxide	unsaturated lipids	unsaturated bonds	black
Copper – rubeanic acid (Holczinger 1959)	free fatty acids	carboxylic	greenish-black
Long Ziehl Neelsen (Lison 1960)	"lipofuscin"	"acid resistant basophilia"	magenta

TABLE 1 *Histochemical Reactions Performed for the Demonstration of Nucleic Acids*

Reaction	Macromolecule demonstrated	Chemically reactive group	Colour of final reaction product
Gallocyanin chromalum (Einarson 1951)	DNA and RNA	phosphate	deep blue
Gallocyanin chromalum following RNA ase digestion	DNA	phosphate	deep blue
Methyl green pyronin (Brachet 1942)	DNA (+RNA)	phosphate	blue green (DNA) red (RNA)
Feulgen's nucleal (Feulgen & Rossenbeck 1924)	DNA	deoxyribose	magenta

TABLE 2 *Histochemical Reactions Performed for the Demonstration of Proteins*

Reaction	Macromolecule demonstrated	Chemically reactive group	Colour of final reaction product
Fast green FCF (pH 8) following trichloroacetic acid extraction (Alfert & Geschwind 1953)	basic proteins	cations (amino)	green
Fast green FCF (pH 8) without trichloroacetic acid extraction	unmasked basic proteins	cations (amino)	green
Azure A-eosin B (Lillie 1965)	proteins, nucleic acids	anions and cations (carboxylate, phosphate, amino)	red, blue or mixtures of these colours
Ninhydrin/Schiff (Yasuma & Itchimawa 1953)	proteins containing lysine	ε-amino	magenta
Dichloronaphthol hypochlorite (Deitch 1961)	proteins containing arginine	guanidine	orange red
Diazotizationcoupling (Glenner & Lillie 1959)	proteins containing tyrosine	p-hydroxyphenyl	purplish red
Dimethylaminobenzaldehyde-nitrite (DMAB nitrite) (Adams 1957)	proteins containing tryptophan	indole	blue
Dihydroxy dinaphthyl disulphide (DDD) *with preceding* reduction (Barnett & Seligman 1952)	proteins containing *cystine and cysteine*	sulphydryl	reddish pink
Dihydroxy dinaphthyl disulphide (DDD) without preceding reduction (Barnett & Seligman 1952)	proteins containing cysteine	sulphydryl	reddish pink

As a control group for these 7 biopsies 12 of the 102 liver biopsies serving as control group for the paraffin section were available as formol calcium fixed material.

The histochemical reactions performed for nucleic acids, proteins carbohydrates, lipids, and enzymes are listed in Tables 1–5 along with information on the macromolecule demonstrated, the chemically reactive group, and the colour of the final reaction product.

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TABLE 7 *Results of Histochemical Reactions for the Demonstration of Proteins as Applied to Mallory Bodies*

Reaction	—	0	+	+ +	+ + +
Fast green FCF (pH 8) following trichloracetic acid extraction	7			25	2
Fast green FCF (pH 8) without trichloracetic acid extraction	6			26	2
Azure A eosin B pH 4	4			7a)	23a)
Azure A eosin B pH 5	2			8a)	24a)
Azure A eosin B pH 6	5		19b)	10b)	
Azure A eosin B pH 7	5		11b)	18b)	
Azure A eosin B pH 8	6		7b)	19b)	2b)
Ninhydrin/Schiff	5		10	14	5
Dichloronapthol hypochlorite	2		8	21	3
Diazotization coupling	2		7	15	10
DMAB-nitrite	5	1	3	13	12
DDD with preceding reduction	14		9	11	
DDD without preceding reduction	12		14	8	

a) acidophilic
b) basophilic

Proteins

Table 7 shows the results of the methods designed for the demonstration of protein as applied to Mallory bodies

With the fast green FCF method the Mallory bodies showed a moderately positive reaction in 25 cases and a strong positive reaction in 2 cases

The reaction demonstrated basic protein, and it may be noted that this is in amounts nearly comparable to what is normally seen in the nuclei

In order to decide whether the protein is in a free or bound condition, the above reaction has been modified by excluding the trichloroacetic acid extraction of nucleic acids The method showed moderately positive reaction in 26 cases and strongly positive reaction in 2 cases

These results are practically the same as the results obtained by the classical Alfert & Geshwind method It is thus shown, that the basic protein in Mallory bodies is primarily unconjugated with nucleic acid in contrast to the basic protein of both cytoplasm and nuclei of the liver cells which is unstained with the modified method Furthermore, it should be noted that red cells with both methods give a strongly positive reaction which compares favourably with the fact that they contain large amounts of unconjugated basic protein

The staining with azure A eosin B at five different pH values is designed to give an impression of the apparent isoelectric point of the total sum of macromolecules present in the Mallory bodies The result shows that the Mallory bodies are acidophilic at pH values below 5 and basophilic at pH values above 6 The apparent isoelectric point according to this reaction is thus between pH 5 and 6

The reaction of the Mallory bodies with both fast green FCF and azure A-eosin B is particulate in the majority of cases

The following six reactions demonstrate specific protein bound amino acid residues The ninhydrin/Schiff reaction shows that Mallory bodies contain moderate amounts of lysine, while Deitch's method shows that they contain small amounts of arginine Glenner & Lillie's and Adams' methods show considerable amounts of tyrosine and tryptophan respectively It is noteworthy that one biopsy with a typical Mallory body shows no reaction for tryptophan Barnett & Seligman's method shows a weakly to moderately posi-

TABLE 5 *Histochemical Reactions for the Demonstration of Enzyme Activity*

Reaction	Substrate	Colour of final reaction product
Alkaline phosphatase (Pearse 1954) simultaneous coupling azo dye method for	*a* naphthyl phosphate	red
Acid phosphatase (Barka 1960) simultaneous coupling azo dye method for	a naphthyl phosphate	reddish brown
Adenosine triphosphatase (Wachstein & Meisel 1957) lead method for	adenosine triphosphate	black
Esterase (Davis & Ornstein 1959) simultaneous coupling azo dye method for	*a* naphthyl acetate	reddish brown
NADH diaphorase (Scarpelli et al 1958) tetrazolium method for	nitro BT	blue

formed The following designations have been used

- — Mallory bodies not identified or reaction not performed
- O no reaction
- \+ weak reaction
- \+ + moderate reaction
- \+ + + strong reaction

RESULTS

Nucleic Acids

Table 6 shows the results of the methods employed for the demonstration of nucleic acids as applied to the Mallory bodies

With the gallocyanin chromalum reaction a strong positive reaction was achieved in 2 cases a moderately strong reaction in 18 cases, and a weakly positive reaction in 8 cases

Very similar results are obtained with the methyl green pyronin method This reaction is highly unspecific but does, however give *an indication* of the distribution of nucleic acids, as deoxyribonucleic acid is stained green with methyl green and ribonucleic acid red with pyronin As the Mallory bodies are stained red without any tinge of green it is reasonable to conclude that they contain ribonucleic acid but no deoxyribonucleic acid This is confirmed by the considerably more selective gallocyanin chromalum method which demonstrates both *nucleic acids* With this method a majority of Mallory bodies exhibit a moderately strong reaction while all reaction is eliminated if the sections have been pretreated with RNA ase The reaction in the Mallory bodies is particulate with both pyronin and gallocyanine

Further confirmation to the above conclusion is added by Feulgen s nucleal reaction which shows that the Mallory bodies are devoid of deoxyribonucleic acid

TABLE 6 *Results of Histochemical Reactions for the Demonstration of Nucleic Acids as Applied to Mallory Bodies*

Reaction		0	+	+ +	+ + +
Gallocyanin chromalum	6		8	18	2
Gallocyanin chromalum following RNA ase	8	26			
Methyl green pyronin (pyroninophilia)	14		3	12	5
Feulgen s nucleal	9	25			

TABLE 10 *Results of Histochemical Reactions for the Demonstration of Enzyme Activity as Applied to Mallory Bodies*

Reaction	—	0	+	++	+++
Alkaline phosphatase	3	4			
Acid phosphatase	1		3	2	1
Adenosine triphosphatase	4	3			
Esterase	2		1	2	2
NADH-diaphorase				2	

they contain hydrophobic lipids This is supported by a strongly positive reaction of the Mallory bodies in one case and a moderately positive reaction in three cases by the Sudan black B method In all four cases the reaction of the Mallory bodies was particulate The reaction of the Mallory bodies with both OTAN and Sudan black B were of an intensity comparable to that normally seen in the cytoplasm of liver cells

Enzyme Activity

The results of the histochemical reactions performed for the demonstration of enzyme activity in the Mallory bodies are given in Table 10 No activity of alkaline phosphatase or adenosine triphosphatase was demonstrated in the Mallory bodies On the other hand acid phosphatase activity was shown in the Mallory bodies in six cases The reaction was on the whole of an intensity as that encountered in the lysosomes which usually are situated in the pericanicular portion of the cytoplasm of the liver cell It is of interest that Mallory bodies normally show no predilection for the pericanicular portion of the cell In one case no Mallory bodies could be identified in the section nor in the neighbouring haematoxylin and eosin stained section Esterase activity of varying intensity was found in five cases The intensity was comparable to that usually seen in the cytoplasm of liver cells

NADH diaphorase activity was demonstrated in the Mallory bodies in two cases The intensity was comparable to that usually seen in the cytoplasm of liver cells In five cases the reaction failed completely, probably on account of the fixation procedure which is not ideal for the subsequent demonstration of oxidative enzymes, and these cases have therefore been omitted from the table

The Mallory bodies in our material thus contain ribonucleic acid, unconjugated basic protein, glycogen, a diastase resistant PAS-positive material, and hydrophilic lipids In addition they exhibit NADH diaphorase, acid phosphatase and esterase activity

DISCUSSION

Nucleic Acids and Proteins

Our results are in accordance with those found by *Norkin et al* (1960) and *Becker* (1961) with the following exceptions *Norkin* was unable to demonstrate tryptophan, and *Becker* did not find either tryptophan or tyrosine

Our results with the azure A eosin B procedure at different pH values show that the Mallory bodies are acidophilic at pH lower than 5 and basophilic at pH values above 6 The acidophilia is due, as the strong affinity for fast green FCF at pH 8 shows, to the presence of basic protein containing positively charged amino, guanidyl and possibly histidyl groups The basophilia is, on the other hand, due to negatively charged phosphate groups in ribonucleic acid and probably carboxylate groups in protein

Carbohydrates

That the PAS reaction of the Mallory bodies in all cases is reduced in intensity following predigestion with diastase speaks strongly in favour of the hypothesis that a

TABLE 8 *Results of Histochemical Reactions for the Demonstration of Carbohydrates as Applied to Mallory Bodies*

Reaction	—	0	+	+ +	+ + +
PAS	8		3	9	14
PAS following diastase	8	13	8	5	
Aldehyde fuchsin pH 1	7	27			
Alcian blue pH 1	8	26			
Alcian blue pH 2 5	8	26			

tive reaction for both sulphydryl and disulphide groups

Table 8 shows the results of the methods selected for the demonstration of carbohydrates as applied to Mallory bodies

With the periodic acid Schiff (PAS) method the Mallory bodies in 14 cases exhibited a *strongly* positive reaction, in 9 cases a moderately positive reaction, and in 3 cases a weakly positive reaction On the whole, the reaction of the Mallory bodies was stronger than the normal cytoplasmic reaction due to glycogen

When the PAS reaction was performed following digestion with diastase for one hour at 37° C Mallory bodies in 5 cases exhibited a moderately positive reaction and in 8 cases a weakly positive reaction In 13 cases Mallory bodies that gave no reaction were found In all cases there was a reduction in the intensity of the reaction of the Mallory bodies The reaction could be completely abolished in all Mallory bodies in all sections when these prior to the PAS reaction were subjected to prolonged (48 hours) digestion with diastase

Both with the aldehyde fuchsin and alcian blue methods the Mallory bodies give no reaction These results show that the Mallory bodies do not contain acid mucopolysaccharides or acid mucoproteins.

The Mallory bodies in our material thus contain a PAS positive substance which may be partly removed by digestion with diastase This suggests that some of the material may be glycogen Methods for the demonstration of acid mucopolysaccharides and acid mucoproteins did in no case give a positive reaction A further interpretation of the results will be given in the discussion

Lipids

The results of the histochemical reactions performed for the demonstration of lipids in the Mallory bodies are given in Table 9 The negative reactions with oil red O osmium tetroxide and rubeanic acid clearly show that no hydrophobic lipids are present in the Mallory bodies On the other hand the positive red reaction of the Mallory bodies in four cases with the OTAN method indicates that

TABLE 9 *Results of Histochemical Reactions for the Demonstration of Lipids as Applied to Mallory Bodies*

Reaction	—	0	+	+ +	+ + +
Oil red O	3	4			
Sudan black B	3			3	1
OTAN { black	3	4			
OTAN { red	3		1	1	2
Osmium tetroxide	4	3			
Copper rubeanic acid	3	4			
Long Ziehl Neelsen	4	3			

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substantial part of the PAS-positive material may be glycogen That the Mallory bodies are devoid of all PAS-positive material following 48 hours diastase digestion can, however, hardly be taken in support of a view according to which all the PAS positive material is considered to be glycogen, as this result may easily be due to the fact that a PAS-positive material other than glycogen simply may be dissolved A content of acid mucopolysaccharide or acid mucoprotein can be ruled out as the Mallory bodies fail to give a positive reaction with either alcian blue or aldehyde fuchsin This still leaves the possibility open, that the remaining PAS-positive material in Mallory bodies following diastase digestion may be due to neutral mucopolysaccharides, glycoproteins, neutral mucoproteins, glycolipids or unsaturated polymerized lipids of the nature of lipofuscin The negative long Ziehl-Neelsen reaction probably excludes the last possibility A final solution of the above problem can only be reached by the use of more refined histochemical methods, and for a project of this kind there were too few sections available from the biopsies

Neither *Norkin* (1960) nor *Becker* (1961) have found PAS-positive material in Mallory bodies, but *Roque* (1953) is of the opinion that they contain glycoproteins The results of the present study are compatible with the latter view

Lipids

The Mallory bodies exhibited NADH-Sudan black B positive material in the Mallory bodies This material binds α-naphthylamine in the OTAN procedure and is most probably phospholipid derived from cellular membranes These results are at least partly in accordance with the results obtained by *Norkin et al* (1960) who found Mallory bodies Sudan black negative but acid haematein positive

Enzyme Activity

The Mallory bodies exhibited NADH-diaphorase activity, acid phosphatase activity and esterase activity which points to probable mitochondrial, lysosomal and endoplasmic reticular components These results do not harmonize with those described by *Norkin et al* (1960) who found no enzyme activity whatsoever However, it should perhaps be taken into consideration that, while our material has been obtained by biopsy from living patients, *Norkin* has used material obtained at autopsy when a considerable reduction in the enzyme activity may have taken place

In conclusion all Mallory bodies in our material contain the following components though in somewhat varying amounts unconjugated basic protein, ribonucleic acid, glycogen and some other non-acid carbohydrate components as well as phospholipid Further NADH diaphorase, acid phosphatase and esterase activity has been demonstrated It has further been noted that the reactions for ribonucleic acid, protein and phospholipid were particulate in several of the Mallory bodies On the basis of these findings it is our opinion that the Mallory bodies are derived from normal intracytoplasmic structures which are more or less decomposed These structures would seem to include mitochondria, lysosomes, ribosomes and endoplasmic reticulum It would seem to be presumptuous to conclude anything definite as regards stage of development of the Mallory body in relation to the intensity of the different histochemical reactions or the particulate nature of the reaction

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theoretical interest, but also clinical value, as antimetabolites are used for immune suppression in transplantation surgery and in some autoimmune diseases

MATERIAL AND METHODS

A total of 189 male guinea pigs were used The animals were fed on cabbage, turnips, carrots and vitamin pellets 136 animals were treated with 5 fluorodeoxyuridine (5 FUdR), dissolved in 0 9 per cent saline to a concentration of 50 mg per ml The controls (53 animals) were injected with 0 5 ml 0 9 per cent saline The injections were made intraperitoneally The animals were used in three different experiments

In the first test experiment 30 guinea pigs were treated with 5 FUdR in three different doses, 150 mg 15 mg and 1 5 mg per kg body weight The two lower doses were given four times, every 12 hrs on two consecutive days The largest dose 150 mg per kg body weight, caused a high mortality in our animals and was therefore given only twice The animals were investigated two days after the last injection The adrenals, thymus, spleen cervical and mesenterial lymph nodes were dissected out quantitatively and weighed

In the second experiment 56 animals were treated with 5 FUdR in a single dose of 100 mg per kg body weight The animals were dissected after 2 4 8 and 16 days The adrenals and the thymo-lymphatic organs were dissected out quantitatively and weighed The thymic veno-arterial difference in number of lymphocytes was investigated in 42 animals

In the third experiment 32 animals were treated with 5 FUdR in two doses of each 100 mg per kg body weight given with an interval of 24 hrs The animals were dissected two days after the last injection The adrenals and the thymo lymphatic organs were dissected out and weighed The thymic veno-arterial difference in number of lymphocytes was investigated in 17 animals The blood flow through the thymus was determined in 15 animals

The controls were dissected two days after the last injection of saline The adrenals and the thymo-lymphatic organs were dissected out and weighed The thymic veno-arterial difference in number of lymphocytes was investigated in 37 animals The thymic blood flow was determined in 23 animals

The white cell count in blood samples from a thymic vein and a carotid artery was determined in a haemocytometer The lymphocytes were differentiated according to their mitochondrial content in supravital preparations of blood from the thymic vein and the carotid artery This differentiation was performed in a light microscope at a magnification of 1000 × (for details, see *Ernstrom* & *Larsson* 1966) Lymphocytes with 0–15 mitochondria and with above 15 mitochondria are denoted as small and large lymphocytes, respectively, as the mitochondrial content of the lymphocytes is correlated to the size of the lymphocyte (*Wiseman* 1931, *Fichtelius* & *Larsson* 1961, *Ernstrom* & *Larsson* 1963) The difference between the content of lymphocytes in the blood from the thymic vein and the thymic artery was calculated.

The thymic blood flow was determined according to *Larsson* (1966) The thymic release of lymphocytes per min was calculated from the blood flow per min and the thymic veno-arterial difference in number of lymphocytes. The standard deviation of the mean product was obtained from the formula

$$S_{y\bar{x}} = \pm \sqrt{x^2 \quad S^{2}_{y} + y^2 \quad S^{2}_{x}}$$

The results were analysed statistically by Student s *t* test The comparison between the number of lymphocytes of different categories in thymic vein and carotid artery blood was performed by statistical analysis of all the differences in the individual animals The p values < 0.05, < 0.01 and < 0.001 are denoted as almost significant, significant and highly significant respectively

RESULTS

In the first experiment the effect of different doses of 5 FUdR given every 12 hrs over 2 days was tested The lower doses of 1 5 and 15 mg per kg body weight did not cause any mortality, and did not change the body weight nor decrease the organ weights of the treated animals An increased weight of the mesenterial lymph nodes was registered after 15 mg The high dose of 150 mg per kg body weight caused the death of 6 animals out of 10 and decreased the body weight of the 4 surviving animals The weights of thymus, spleen, cervical and mesenterial lymph nodes were markedly reduced (Figs 1–2) The adrenal weight was increased by 18 per cent ($p < 0.05$) No other dose of 5-FUdR in the present experiment changed the adrenal weight to a statistically significant extent

In the second experiment a single dose of 100 mg 5 FUdR was given The animals were investigated at different intervals from 2 to 16 days after the treatment All animals sur

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THYMIC RELEASE OF LYMPHOCYTES IN GUINEA-PIGS TREATED WITH 5-FLUORODEOXYURIDINE

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Guinea pigs were treated with different doses of 5 FUdR and investigated at different intervals from the treatment The weight of the different lymphoid organs was registered The release of lymphocytes from the thymus was determined by comparison between the content of lymphocytes in blood from the thymic vein and from the carotid artery In one group the blood flow through the thymus was determined and the total output of lymphocytes from the thymus per min was calculated and compared to that in normal animals The results showed that, among the lymphoid organs the thymus was the one most sensitive to 5 FUdR No regeneration of thymic weight was seen within 16 days Both spleen and cervical lymph nodes showed initial atrophy followed by a rapid recovery The release of thymic lymphocytes was abolished 2 days after a single dose of 5 FUdR At 4 days a release of both small and large cells occurred At 8 and 16 days again no export occurred The release of lymphocytes after 4 days coincided with the rapid regeneration of the spleen and lymph nodes It is suggested that the release of premature lymphocytes from the involuted thymus is due to a regulative feed back mechanism from the non thymic lymphoid tissue depleted of lymphocytes

Antimetabolites are preferentially used in tumour therapy due to their ability to inhibit cell proliferation However, all rapidly proliferating organs are affected by treatment with drugs which interfere with nucleic acid synthesis Among the thymolymphatic organs the most intense cell proliferation occurs in the thymus of young individuals and in accordance with this the thymus is more sensitive to such drugs than other lymphoid organs (*Pierce* & *Varco* 1963, *Kovalev* 1966) Functionally, this kind of antimetabolite causes a depression of the immune response (see *i e Schwartz et al* 1958) It has been suggested that this depression might essentially be due to the effect on the thymus (*Pierce* & *Varco* 1963) Evidence is accumulating that the thymic release of lymphocytes to the blood and export of cells to other parts of the body is essential for the cellular immune response and important also for the humoral response The aim of the present investigation is to study the capacity of the thymus to release lymphocytes to the blood after treatment with the pyrimidine analogue, 5 fluorodeoxyuridine, known to interfere with DNA synthesis (*Heidelberg et al* 1957) and to cause immune suppression (*Fisher et al* 1966, *Laurentaci* & *Monosi* 1967) Such detailed knowledge has not only

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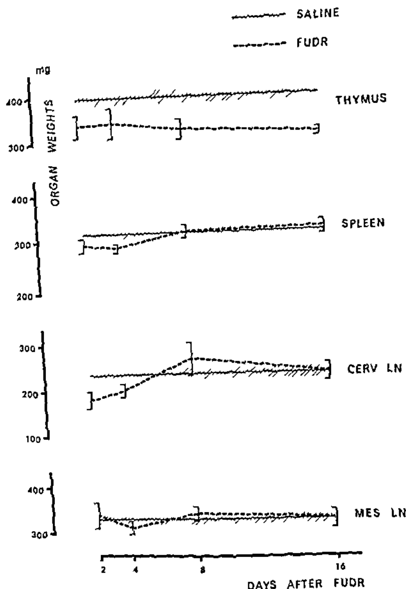


Fig 3 Weight of the thymus spleen cervical and mesenteral lymph nodes at different intervals after treatment with 100 mg 5 FUdR per kg body weight The hatched area indicates the organ weights of control animals injected with saline Mean ± standard deviation of the mean

again (Fig 4) An increased number of granulocytes was found 8 days after treatment with 5 FUdR (Fig 4)

The thymic veno-arterial difference in number of lymphocytes was determined Two days after 100 mg 5 FUdR per kg body weight no significant release of lymphocytes from the thymus was found (Table 1) After 4 days a release of lymphocytes of all categories was demonstrated (Table 1) Later, after 8 and 16 days, the thymic release of lymphocytes was again totally abolished

In the third experiment a dose of 100 mg 5 FUdR per kg body weight was given twice

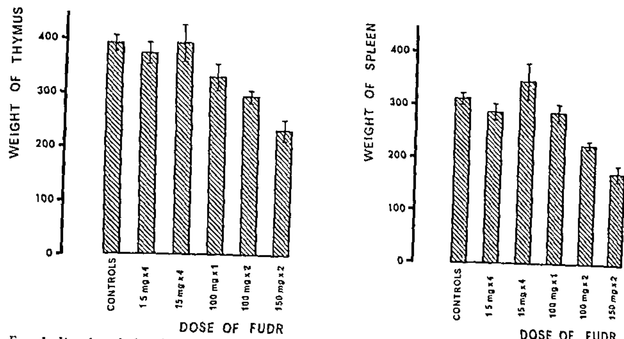


Fig 1 Weight of the thymus and spleen after treatment with different doses of 5 FUdR The control animals were treated with saline Mean ± standard deviation of the mean

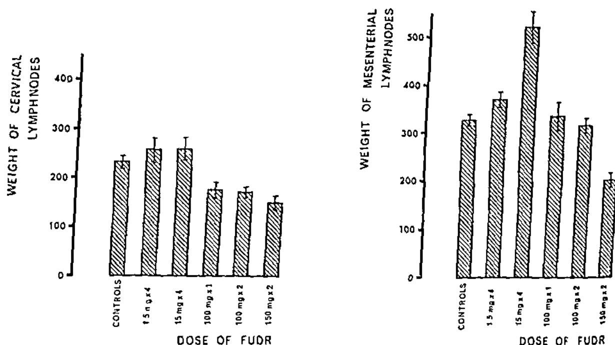


Fig 2 Weight of the cervical and mesenterial lymph nodes after treatment with different doses of 5 FUdR The control animals were treated with saline Mean ± standard deviation of the mean

vived and the body weight was not decreased This dose caused a significant decrease of the thymus weight, which was about 80 per cent of the control weight during the whole period investigated (Fig 3) The weight of the cervical lymph nodes was slightly decreased 2 and 4 days after treatment with 5 FUdR ($p<0.05$) but no mal again 8 and 16 days afterwards (Fig 3) The weights of the spleen (Fig 3) and mesenterial lymph nodes (Fig 3) were not significantly changed

The treatment resulted in lymphopenia in the blood after 2 and 4 days *After 8 and 16* days the lymphocyte number was normal

TABLE 2 *Calculation of the Export of Lymphocytes from One Thymic Lobe in Guinea Pigs 2 Days after the Last of Two Injections of 5 FUdR (100 mg per kg Body Weight) or Saline*

	Thymic blood flow (µl per min)	Thymic veno-arterial difference in number of lymphocytes (cells per µl)			Thymic export of lymphocytes (cells per min)		
		Total no	No of cells with ≦ 15 mitochondria	No of cells with > 15 mitochondria	Total no	No of cells with ≦ 15 mitochondria	No of cells with > 15 mitochondria
5-FUdR	32 4 ± 5 4 n = 14	348 ± 77 p < 0 001	199 ± 76 p < 0 05	149 ± 35 p < 0 001	11275 ± 2502 p < 0.001	6458 ± 1329 p < 0 001	4815 ± 1382 p < 0 01
saline	49 9 ± 4 3 n = 23	479 ± 98 p < 0 001	453 ± 118 p < 0 01	25 ± 45 —	23878 ± 5306 p < 0 001	22618 ± 6182 p < 0 01	1260 ± 7251 —

The calculation is based on the values of the thymic blood flow and the thymic veno-arterial difference in number of lymphocytes The lymphocytes are subdivided in cells with ≦ 15 mitochondria (smaller lymphocytes) and cells with > 15 mitochondria (larger lymphocytes) A reduced though still significant export of lymphocytes is demonstrated after treatment with 5 FUdR The reduction occurs among the smaller lymphocytes A considerable and significant export of larger lymphocytes is demonstrated after treatment with 5 FUdR. No such export of larger cells occurs in normal animals

port of lymphocytes from the thymus was decreased by 39 per cent, as judged by the thymic veno arterial difference in number of lymphocytes A significant decrease in the thymic blood flow was also registered (Table 2) The combination of the veno arterial difference and the blood flow permits a calculation of the export of cells per min The treatment with 5 FUdR caused a decrease to 47 per cent of the normal thymic release of lymphocytes per min (Table 2)

DISCUSSION

In the present dose experiments the smaller doses of 5 FUdR had no effect on the weights of the different thymo-lymphatic organs An exception was the increase in weight seen in the mesenterial lymph nodes after 15 mg per kg body weight given 4 times (Fig 2) No explanation of this hypertrophy is suggested

The larger doses caused increasing atrophy of the thymo lymphatic organs Among the organs investigated the thymus was found to be the most sensitive to 5 FUdR, which is in agreement with earlier reports on 5 FU and 6-mercaptopurine (*Kovalev* 1966, *Miller & Cole* 1967) After a single dose of 100 mg 5 FUdR per kg body weight a pronounced thymic atrophy occurred and no regeneration of thymic weight occurred within the 16 days of the experiment The spleen and lymph nodes showed a smaller initial decrease in weight followed by a rapid regeneration to normal weight after 8 days The least sensitive of the lymphoid organs were the mesenterial lymph nodes

The normal thymus releases small lymphocytes characterized by low mitochondrial content Two days after treatment with 5 FUdR this thymic release was abolished However, after 4 days a release of cells had recurred, and this release included not only small lymphocytes with low mitochondrial content, but also large lymphocytes with high mitochondrial content The same result was found in the guinea pigs which were treated with two doses of 5-FUdR and investigated 2 days after the last dose This thymic release of lymphocytes was followed by a completely abolished export of lymphocytes of all categories from the thymus 8 and 16 days after treatment.

The release of cells from the *involuted* thymus 4 days after 5 FUdR coincides in time with the rapid regeneration of the spleen and cervical lymph nodes The same kind of coincidence between release of lymphocytes from an involuted thymus and rapid regeneration of non thymic lymphoid organs in irradiated

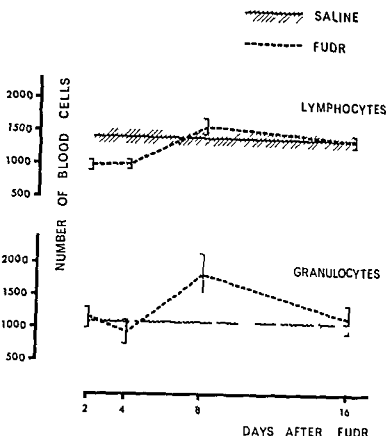


Fig 4 Number of lymphocytes and granulocytes per µl of carotid artery blood at different intervals after treatment with 100 mg 5 FUdR per kg body weight The hatched area indicates the cell number in blood from control animals treated with saline Mean ± standard deviation of the mean

TABLE 1 *Thymic Veno Arterial Difference in Number of Lymphocytes per µl of Blood in Guinea Pigs at Different Intervals after a Single Injection of 5 FUdR in a Dose of 100 mg per kg Body Weight Mean ± Standard Deviation of the Mean*

	Days after 5 FUdR	No of animals	Thymic veno-arterial difference in number of lymphocytes (cells per µl)		
			Total no	No of cells with ≦15 mitochondria	No of cells with >15 mitochondria
Controls	-	38	209±70 p<0 05	208±68 p<0 01	1±57 —
5 FUdR	2	12	45±73 —	54±60 —	—10±21 —
5 FUdR	4	9	257±82 p<0 05	188±58 p<0 01	69±29 p<0 05
5 FUdR	8	11	—10±81 —	—24±79 —	14±21 —
5 FUdR	16	10	—105±138 —	—93±124 —	—13±25 —

at a 24 hour interval The animals were investigated 2 days after the last injection The mortality was 10/42 The mean body weight of the survivors decreased with 15 per cent The weight of the thymus cervical lymph nodes and the spleen decreased significantly while the weight of the mesenterial lymph nodes was unchanged (Figs 1–2) The ex

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KARYOTYPIC PROFILE ALTERATIONS IN EHRLICH ASCITES TUMOUR CELLS DURING DEVELOPMENT OF RESISTANCE TO DAUNORUBICINE

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Karyotype determinations were made on a wild Ehrlich ascites tumour and on a subline which was made resistant to daunorubicine (DNR) by long term treatment with this drug. During the development of resistance, karyotypic alteration occurred, with a change from near tetraploidy to hyperdiploidy and the appearance of marker chromosomes which were not found in the original tumour. The resistance was gradually lost on cessation of DNR treatment. A tumour subline which, after having been resistant, had become sensitive again presented a karyotype resembling that of the original tumour. Another tumour which had partially lost its resistance, was composed of a mixture of cells with karyotypes characteristic either of the sensitive or of the resistant tumour. It had been found previously that the rate of growth in the resistant tumour was lower than that observed in the sensitive one. The loss of resistance was explained by abundant growth of sensitive cells present in the resistant tumour. The possibility of checking loss of resistance clinically by examining the karyotype of the malignant cells is discussed.

Daunorubicine (DNR)* is a cytotoxic antibiotic which plays an important role in the treatment of acute leukaemia (1, 5). The drug belongs to the antracyclines and inhibits the synthesis of both DNA and RNA, supposedly by forming a complex with the DNA of the cells (4, 8). Apart from this, the drug has presumably a direct antimitotic effect (4).

DNR inhibits the growth of a number of animal tumours, including Ehrlich ascites tumour (7). In a previous paper a description has been given as to how resistance to DNR was developed in an Ehrlich ascites tumour by daily administration of the drug in partially tumour inhibiting doses during 16 weekly passages (6). The resistance was maintained during continued treatment with DNR. A subline, in which DNR treatment was discontinued, gradually lost its resistance. Hence, after 8 passages without DNR, it showed partial sensitivity and became then gradually just as sensitive to DNR as the original wild tumour had been.

During the present investigation, chromosome analyses were made of the various sensitive and resistant tumour sublines.

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* Daunorubicine (NSC 82151) is identical with daunomycin and rubidomycin.

guinea pigs has recently been reported (*Ernstrom* 1971) In the present study the forced release of thymic cells resulted in a pronounced and prolonged thymic atrophy

From the present results and from the study of irradiated guinea pigs (*Ernström* 1971), it may be proposed that some kind of mechanism regulating the release of thymic lymphocytes exists A depletion of lymphocytes in spleen and lymph nodes may stimulate the thymus to release available lymphocytes If the thymus is damaged, this stimulation may result in a premature export of larger cells which have not been able to differentiate to small lymphocytes due to the disturbance of the nucleic acid synthesis following irradiation or treatment with antimetabolites like 5 FUdR

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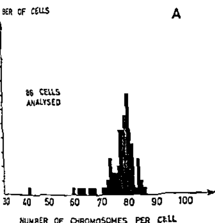
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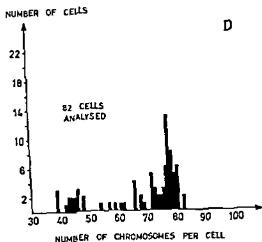
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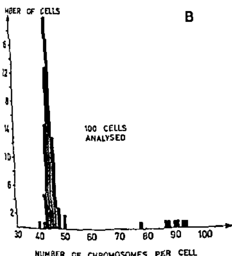
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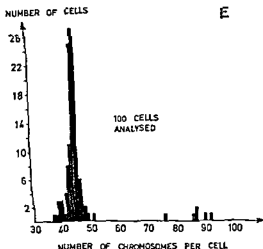
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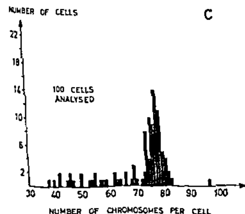


Fig 1 Distribution of chromosome numbers in an Ehrlich ascites tumour and in various DNR sensitive and DNR resistant sublines

A Wild DNR sensitive tumour EHR 2

B DNR resistant tumour EHR 2/DNR +

C Secondarily DNR sensitive tumour 34 passages after discontinuation of the DNR treatment EHR 2/DNR—

D Partially secondarily DNR sensitive tumour 8 passages after discontinuation of the DNR treatment, EHR 2/DNR—

E The same tumour as that described under D but treated with DNR during one passage before sampling EHR 2/DNR—/+

MATERIAL AND METHODS

Mice and Tumours

Mice and tumour strains have been described previously in further detail (6) First generation hybrids of female random bred Swiss mice and *male inbred DBA* mice were used throughout the study The wild tumour was a near tetraploid strain (EHR 2), originally obtained from Microbiological Associates, Inc Bethesda USA Furthermore a subline of this tumour was studied in which resistance to DNR had been developed by treatment with this drug in partially growth inhibiting doses daily for 5 days during 16 weekly passages and in which the resistance was then maintained by DNR treatment (EHR 2/DNR+) Another subline was also studied which after development of resistance, was transplanted without DNR treatment and gradually lost its resistance (EHR 2/DNR—) All tumours were maintained by weekly intraperitoneal inoculations of 0 2 ml undiluted ascites fluid on mice of alternating sex

Drugs and Drug Administration

DNR (as daunomycin hydrochloride, Farmitalia) was supplied by the Cancer Chemotherapy National Service Center National Cancer Institute, Bethesda USA Immediately before administration the drug was dissolved *in an 0 9 per cent NaCl* solution and the dose was injected intraperitoneally as previously described (6)

Chromosome Preparations

Seven days after inoculation of 0 2 ml undiluted ascites fluid, each mouse was given 0 2 ml *i p* of a colcemid (Ciba) solution 0 4 mg/ml Four hours later the mice were sacrificed and two drops of ascites fluid were removed and transferred to *micro test tubes containing* 2 ml of an 0 95 per cent sodium citrate solution The test tubes were then incubated in a water bath at 37 5° C for 25 minutes Upon centrifugation at 110 × g for 10 minutes the cells were fixed by means of a mixture of ethanol and glacial acetic acid (3 1) and stored overnight at a temperature below zero After centrifugation the sediment was resuspended in methanol and glacial acetic acid (3 1) Smears were prepared by using the air drying technique and stained with Giemsa stain

Karyotype Analysis

A total of 80 to 100 cells from each tumour was analysed using a Zeiss *photomicroscope II* Any marker chromosome present was registered during the microscopic examination, and the mitosis was photographed (Kodak Panatomic X) The negatives were projected on to paper the chromosomes were counted and at the same time the marker chromosomes registered were checked The metaphase score in the smears investigated ranged from 0 5 to 2 0 per cent

Test for DNR Sensitivity

The technique used has been described previously (6) A number of mice were inoculated intraperitoneally with 15 × 10^6 tumour cells and were then divided into a treatment group and a control group each comprising 10 12 mice The two groups were given 1 25 mg/kg of DNR *i p* daily for 5 consecutive days beginning 24 hours after the inoculation (the LD_{10} *of the drug (6)*), and 0 2 ml of an 0 9 per cent NaCl solution respectively, identical methods of administration and dosage scheme being employed Seven days after the inoculation the mice were sacrificed the ascites fluid was removed and the total ascites cell volume in each mouse was found by measuring the volume of ascites fluid and determining the cell volume percentage by centrifugation in a haematocrit centrifuge The degree of inhibition by DNR was expressed as the average total tumour cell volume per mouse in per cent of the corresponding unit found in the controls

Frozen Storage of Tumours

Some analyses were made on tumour strains which had been stored at 194° C in liqu d nitrogen for periods of up to 18 months The specimens were frozen using a modification of the method described by *Kline et al* (12) The tumour cells were suspended in a mixture of 80 per cent Eagle s Basal Medium 15 per cent bovine serum and 5 per cent dimethyl sulphoxide with about 100 × 10^5 cells/ml and were frozen at a rate of 2 5–3 5° C/minute in a Linde liquid nitrogen refrigerator Thawing was done rapidly by immersion in a water bath at 37° C Then 0 2 ml suspension was inoculated in each of four mice In all cases a 100 per cent take was obtained The chromosome analyses were made at the earliest after a single passage of one week in mice The karyotype of EHR 2 tumour cells which had been frozen for two years was compared with karyotypes of cells from the same tumour maintained exclusively in mice The karyotype of the former could not be distinguished from that of the latter No changes in the DNR sensitivity of the various tumours were demonstrated after frozen storage

RESULTS

As appears from Fig 1 the wild DNR sensitive EHR 2 tumour consisted mainly of near tetraploid cells with chromosome numbers around 81, with a fairly wide variation Only 1 *per cent* of the cells investigated had

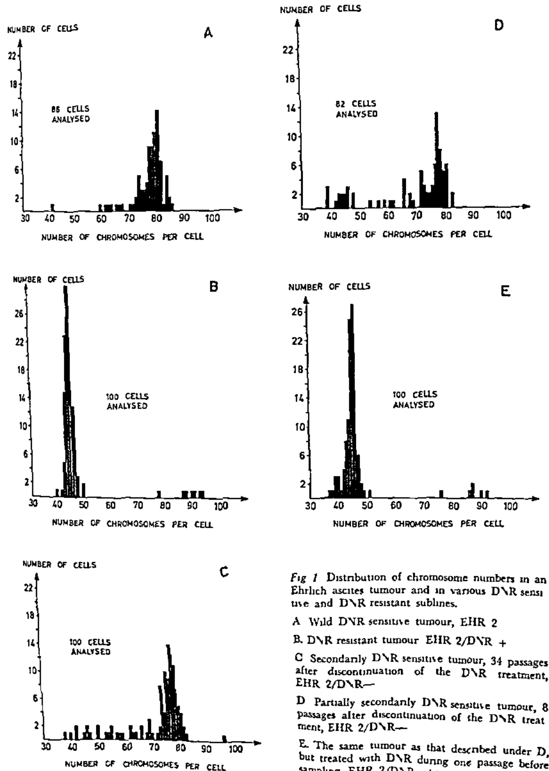


Fig 1 Distribution of chromosome numbers in an Ehrlich ascites tumour and in various DNR sensitive and DNR resistant sublines.

A Wild DNR sensitive tumour, EHR 2

B. DNR resistant tumour EHR 2/DNR +

C Secondarily DNR sensitive tumour, 34 passages after discontinuation of the DNR treatment, EHR 2/DNR—

D Partially secondarily DNR sensitive tumour, 8 passages after discontinuation of the DNR treatment, EHR 2/DNR—

E. The same tumour as that described under D, but treated with DNR during one passage before sampling EHR 2/DNR—/+

TABLE 1 *Response to Daunorubicine (DNR) Therapy* in vivo *and Marker Chromosome Combination of an Ehrlich Ascites Tumour and of Various DNR-Sensitive and DNR Resistant Sublines of This Tumour*

Tumour	Number of passages after development of resistance to DNR	Response to treatment Tumour growth in % of control after DNR treatment, 1 25 mg/kg i p daily for 5 days*	Number of cells analysed	Chromosome analyses % of cells with one or more of the marker chromosomes listed					
				I alone	I + II	I + III	I + (IV and/or V)	No marker chromosomes	Other combinations
EHR 2		16 ± 9	86	80 †	13 †	3 †	0	2	2
EHR 2/DNR +	29	93 ± 13	100	1 §	0	0	99 §	0	0
EHR 2/DNR—	34	1 0 ± 0 2	100	92 †	0	2 †	0	5	1
EHR 2/DNR—	8	47 ± 7	82	29 †	0	54 †	15 §	1	1
EHR 2/DNR—/ +	9		100	3 §	0	0	96 §	0	1

The individual marker chromosomes are shown in Fig 2
* Mean ± standard deviation on the mean
§ The cells mainly hyperdiploid
† The cells mainly near tetraploid

chromosome numbers below 60 In the majority of cells a large metacentric marker chromosome appeared, shown as I in Fig 2 Furthermore a smaller metacentric marker chromosome was found, shown as II in Fig 2 A small proportion of the cells contained a large submetacentric marker chromosome, shown as III in Fig 2 The distribution of the marker chromosomes appears in Table 1 *It will be seen that the tumour cells* analysed could be classified mainly as one of two types near tetraploid cells with only marker chromosome I and near tetraploid cells with both marker chromosomes I and II Many of the cells contained two or more marker chromosomes I

The DNR resistant tumour EHR 2/DNR+ was investigated after DNR treatment over a total of 45 passages, no DNR being given *during the last passage before examination* The majority of the cells had chromosome numbers around 46 as shown in Fig 1 Most of the cells contained marker chromosome I Marker chromosomes II and III were not observed but an acrocentric marker chromosome with a double achromatic lesion was found shown as IV in Fig 2 and an acrocentric marker chromosome larger than all the other chromosomes of the cells shown as V in Fig 2 The distribution of the marker chromosomes appears in Table 1 Nearly all the cells could be classified as hyperdiploid cells all containing marker chromosome I and in addition either marker chromosome IV or V or both with almost equal numbers of IV and V and with both IV and V being present in more than 50 per cent of the cells In 6 per cent of the cells analysed the chromosome numbers were within the tetraploid range (78–94) In these cells the combinations of marker chromosomes were identical to those found in the remaining cells of this tumour

The tumour EHR 2/DNR— was investigated 34 passages after discontinuation of the DNR treatment following development of resistance At this stage the tumour had again become sensitive *in vivo* to DNR As shown in Fig 1 and Table 1, nearly all cells presented a karyotype similar to that found in the near tetraploid cells with marker chromosome 1 only, described under EHR 2 However, the variations in chromosome numbers were more pronounced, and 17 per cent of the cells of this type investigated had a chromosome number below 60

The tumour EHR 2/DNR— was also investigated 8 passages after discontinuation of DNR treatment, following development of resistance At this stage the tumour appeared to be partially sensitive *in vivo* to DNR As shown in Fig 1 and Table 1, the cells of this tumour could be classified under three principal types near tetraploid cells with marker chromosome I only, similar to one of the cell types described for EHR 2, hyperdiploid cells with a marker chromosome combination as that found in the cells characteristic of EHR 2/DNR+, and a large group of near tetraploid cells with both marker chromosomes I and III The latter chromosomal pattern was found in a limited proportion of EHR 2 and of the completely sensitive EHR 2/DNR—, whereas it was not observed in EHR 2/DNR+

The EHR 2/DNR— tumour investigated 8 passages after withdrawal of DNR was transplanted to mice which were treated with 1 25 mg/kg of DNR *i p* daily for 5 days, as described under Material and methods As previously mentioned, this dose inhibits almost totally the growth of EHR 2, but does not influence the growth of EHR 2/DNR+ The karyotype of the tumour cells remaining after this treatment is shown as EHR 2/DNR—/+ in Fig 2 and Table 1 from which it appears that now the tumour consists almost exclusively of *hyperdiploid* cells characteristic of EHR 2/DNR+, whereas no cells were observed with a karyotype similar to that found in the two near tetraploid cell types which existed before the passage with DNR treatment There were, however, a few cells with marker chromosome I only but those cells were hyperdiploid

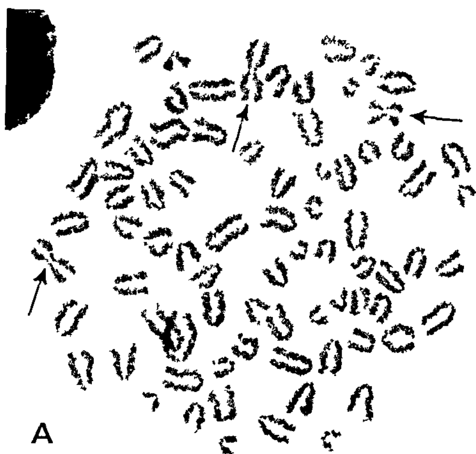
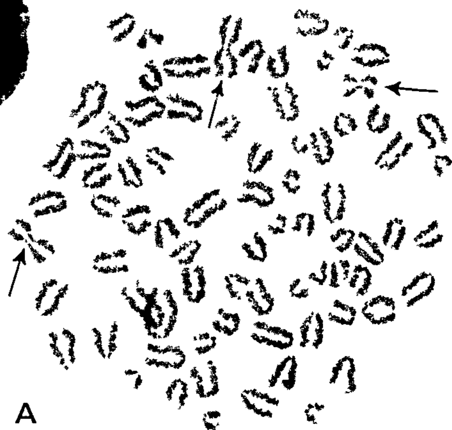




Fig 2


C
D



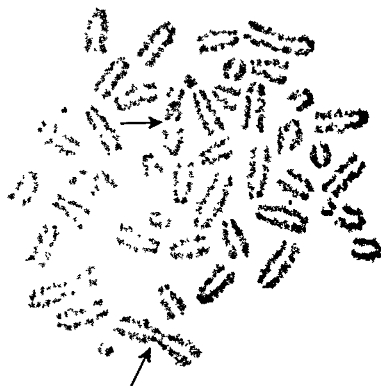


Fig 2.



Fig 2
Karyotypes of Ehrlich ascites tumour cells in which the marker chromosomes are indicated by arrows

A Near tetraploid cell from a wild DNR sensitive tumour EHR 2 Karyotype with 77 chromosomes *two large metacentric marker chromosomes (I) and one small metacentric chromosome (II)*
B Hyperdiploid cell from a DNR resistant tumour EHR 2/DNR + Karyotype with 46 chromosomes, one large metacentric marker chromosome (I) and one acrocentric chromosome with a double achromatic lesion (IV)

C Near tetraploid cell from a secondarily DNR sensitive tumour EHR 2/DNR—, 34 passages without DNR treatment Karyotype with 76 chromosomes two large metacentric marker chromosomes (I)
D Near tetraploid cell from a partially DNR resistant tumour, EHR 2/DNR— 8 passages without DNR treatment Karyotype with 77 chromosomes two large metacentric chromosomes (I) and one large submetacentric marker chromosome (III)

E Hyperdiploid cell from a partially resistant tumour, treated with DNR during one passage before sampling EHR 2/DNR—/+ Karyotype with 46 chromosomes one large metacentric marker chromosome (I) one acrocentric marker chromosome with a double achromatic lesion (IV) and one acrocentric marker chromosome which is larger than all other chromosomes (V)
F Hyperdiploid cell from the same tumour as that described under E Karyotype with 47 chromosomes one large metacentric marker chromosome (I) and one acrocentric marker chromosome with a double achromatic lesion (IV)

G Hypertetraploid cell from the same tumour as that described under E Karyotype with 89 chromosomes two large metacentric marker chromosomes (I) and two acrocentric marker chromosomes with a double achromatic lesion (IV)



Fig. 2

although the relationship between the two alterations was not specific In cases of resistance to actinomycin D, developed *in vitro* in Chinese hamster cells, associated with cross resistance to DNR, *Biedler* & *Riehm* (3) found non specific chromosomal changes

On the basis of our study it cannot be concluded that the change in karyotypic profile should be specific for DNR resistance in Ehrlich ascites tumour In the present case of development of resistance, however, the karyotypic alterations described allow of a classification of the individual cells into DNR sensitive and DNR resistant cells Therefore, it would be appropriate to investigate whether clinical development of resistance to DNR, when the drug is used in acute leukaemia, will manifest itself by the development of cell populations with karyotypes which are characteristic for the individual cases A subsequent loss of resistance might be reflected in disappearance of cells with this karyotype, and determination of karyotype might then be applied to reveal loss of resistance, if any

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The excellent technical assistance of Miss *Inge Strarup* Miss *Anette Jacobsen* and Mr *Jørgen Dusing* is gratefully acknowledged

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DISCUSSION

The majority of the cells from the wild EHR 2 tumour were found to be near tetraploid The chromosome numbers varied greatly, which is in agreement with the findings previously described for other strains of Ehrlich ascites tumour (9, 11, 13) By marker chromosome recording, the cells could be classified under several types with characteristic marker chromosome patterns Two different near-tetraploid cell types were found to make up the majority of the cells As regards this tumour, and the remaining tumours investigated, it is doubtful whether the cells examined are representative of all the cells in the tumour, since it is not known whether the metaphase frequency after colcemid treatment is identical in the various types of cells Therefore, the relative distribution between the various types must be viewed with some reservation

During development of resistance to DNR, the karyotypic profile changed, so that a completely resistant subline consisted almost exclusively of hyperdiploid cells with a marker chromosome combination which was not found in the cells analysed from the wild tumour, whilst the combinations characteristic of the wild tumour were not found in the cells analysed from the resistant tumour A tumour, in which the resistance was lost again after having been present, consisted almost exclusively of cells with chromosome numbers and marker chromosomes similar to those found in one of the main types of cells in the wild tumour Another tumour which showed partial loss of resistance, consisted of a mixture of cells, some of the cells had a chromosomal pattern similar to that found in one of the cell types of the wild tumour, some had a pattern similar to that of the characteristic cells of the resistant tumour, and in many cells the karyotype was the same as that found in a few per cent of cells of the wild tumour, but not in the resistant one After one week s treatment with DNR, the partially sensitive tumour was changed, so that it consisted almost exclusively of cells with a karyotype as that of the resistant tumour These findings show that, in the case examined, the DNR resistance is associated with cells of a characteristic chromosomal pattern (near-diploid cells with marker chromosome I, and either one or both of the marker chromosomes IV and V), whereas at least 3 near tetraploid cell types were found which were sensitive to DNR

It was found previously that *in vivo* resistant tumour cells grew to a lower number of ascites tumour cells than the sensitive ones, 7 days after inoculation of the same number of tumour cells of the two types (after inoculation of 15 million tumour cells, an average of 422 and 672 million of tumour cells were obtained, respectively (6)) This, together with the finding that the formerly resistant tumour line, which had partially lost its resistance, consisted of a mixture of sensitive and resistant cells, leads to the supposition that the loss of resistance found is caused probably by simple abundant growth, following withdrawal of the DNR treatment after development of resistance, of sensitive cells which had remained among the resistant tumour cells

Karyotypic alterations associated with development of resistance to cancer chemotherapeutic drugs in other systems have been described previously Hence, *Hauschka* (10) reported on a number of systems including an Ehrlich tumour, in which, during development of resistance to N methylformide, he found a reduction in chromosome number, but still hypotetraploidy, and a pronounced increase in the incidence of a long telocentric marker chromosome The same author described a change in the transplantable murine lymphosarcoma P 1798 from near tetraploidy to near diploidy during development of resistance to cortisone The relationship between increased dihydrofolate reductase (DHFR) activity and karyotype in methotrexate resistant cells in the transplantable mouse leukaemia L 1210 was studied by *Biedler et al* (2), who found increased DHFR activity associated with disappearance of a subtelocentric chromosome in a number of cases,



Fig 1 Nipple tumour with skin surface at top Papillary projections and glandular crypts with cylindrical epithelium thin stromal strands Htx-eos × 115

epithelium with larger nuclei with prominent nucleoli (Fig 3) The diagnosis was adenoma of the nipple and as the tumour was presumed to be benign the patient was thought to have also another primary tumour still to be detected – The patient deteriorated and died 5 weeks after admission

Necropsy

Malmoe 1054 69 Middle aged slightly icteric woman The residual part of the right breast had been converted into multiple cysts filled with brownish fluid No tumours or cysts were seen in the left breast Two firm lymph nodes which were about ½ cm in diameter and had a white cut surface were found in the right axilla. Similar lymph nodes were found also in the mediastinum and along the abdominal aorta One lymph node 2 3 cm in diameter at the bifurcation of the left lower bronchus contained a tumour that had invaded the lung parenchyma The bronchial mucosa was intact. No other tumour growth was found in the respiratory system The abdominal cavity contained 3 litres of thin, yellowish fluid The liver was enlarged (weight 3550 g) and was studded with rounded, partly coalescent, yellow white nodules up to 5 cm in diameter One small metastasis about ½ cm in diameter, was found in the spleen and another of equal size in the left kidney The red bone marrow in the spinal column, especially in the lower thoracic region, showed yellowish, irregular patches – No other tumour was found in any organ despite a very careful search The stomach and intestines contained fluid and coagulated blood, emanating from an ulcer (3 × 3 cm) with eroded vessels and situated in the lesser curvature of the ventricle This bleeding was the immediate cause of death Parts of the lungs kidneys, myocardium spleen, liver, gastric ulcer hypophysis, both breasts and lymph nodes from axillary, supraclavicular parasternal, mediastinal para-aortal and inguinal regions were examined histologically The right breast contained small remnants of the adenoma No other tumour



Fig 2 Nipple tumour Detail of deeper parts of tumour with dense, fibrous stroma and spaces lined by two-layered epithelium Htx-eos × 115

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ADENOMA OF THE NIPPLE WITH CARCINOMATOUS DEVELOPMENT

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The University Department of Pathology, Malmo General Hospital, Malmo, Sweden

Adenoma of the nipple with carcinomatous development A tumour of the nipple of a 56 year old woman had the characteristics of an adenoma of the nipple and was considered benign in spite of small areas of cellular polymorphism The patient died five weeks later and at necropsy tumour metastases were found in lymph nodes in the right axilla, similar to the nipple tumour Other metastases in lymph nodes liver, left kidney, spleen and spinal column were less differentiated but were apparently secondaries of the nipple tumour No other primary tumour was found at autopsy Adenoma of the nipple is generally considered a benign tumour and this case is the first example of malignant change and general spread

Since 1955, when *Jones* (3) reported the first 5 cases of this tumour, which he named florid papillomatosis of the nipple, approximately 76 cases of the lesion have been published – not only in women (1, 2, 4, 6, 7, 8 9) but also in men (8, 5) and children (5) The tumour has been described under various names such as florid papillomatosis of the nipple, adenoma of the nipple, l adenomatose erosive du mamelon subareolar papillomatosis and papillomatosis of subareolar ducts The benign, non-metastasizing nature of the tumour is stressed in spite of its malignant, erosive, macroscopic appearance and its florid histological picture The case reported below is the first of its kind with a malignant course

CASE REPORT

A 56 year-old housewife, para II, was admitted to Malmoe General Hospital in September 1969 During the previous six months she had had increasing fatigue, anorexia with moderate weight loss and low back pain On admission she was in a poor general condition, slightly jaundiced and exhausted Physical examination revealed an enlarged liver, palpable 10 cm below the costal margin The right nipple had been replaced by a hard, indolent tumour (2 × 1 5 × 1 cm) with a red, eroded surface Some firm lymph nodes ½ 1 cm in diameter were found in the right axilla The patient had observed the tumour for at least 2–3 years The tumour was excised

Biopsy Specimen

(Malmoe 1 14112/69) A rounded well circumscribed tumour with a whitish cut surface The tumour reached the skin surface and had a papillomatous appearance (Fig 1) The deeper parts contained glandular spaces lined by one layer of columnar cells with a layer of flat cells against the prominent basal membrane In most areas there was abundant dense fibrous stroma (Fig 2) The same type of two layered epithelium was seen also over the papillary projections There were small marginal areas with large clear cells in the squamous epithelium of the skin a picture resembling that of Paget s disease of the nipple Parts of the tumour showed spaces filled with rounded cells with a clear cytoplasm and rounded rather uniform nuclei Some mitoses were seen In some [illegible]

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Fig 5 Necropsy Bone marrow metastasis with groups of rather dark cells with moderate polymorphism, diffusely infiltrating cells in fibrous stroma
Htx-eos × 120

DISCUSSION

The appearance of the tumour growth in the right axillary lymph nodes was the same as that of the adenoma of the nipple There were rather highly differentiated adenomatous proliferations in a dense stroma (Fig 4 A) as well as more undifferentiated parts (Fig 4 B) Both had their counterparts in the nipple tumour (Figs 2 3) and can thus hardly be interpreted as anything else but metastases from the nipple tumour The other metastases were less differentiated but their histological type could agree with parts of the nipple tumour and some of the metastases in the right axilla No other primary tumour was revealed at autopsy in spite of a thorough search because of the assumed innocence of the nipple tumour The adenoma of the nipple must have undergone a malignant change and be the origin of metastases.

Duitt et al (1) observed adenoma of the nipple in two women, who afterwards had mammary carcinoma, but they did not consider it proved that the cancer had actually developed from the adenoma They also described a case of adenoma of the nipple with similar tumour structures in the axillary lymph nodes, which they thought were the result of "an embolic involvement of an axillary node by a benign breast lesion" Thus, this case seems to be very like the more differentiated axillary metastases in our case No follow up of their case was reported All other reports of adenoma of the nipple support that it is benign, although cases of very long duration (up to 14 years (9), (2)) and one case with a local recurrence (9) are on record



Fig 6 Necropsy Liver metastasis with irregular strands of cells with marked nuclear polymorphism, inconspicious stroma. Compressed liver tissue at the bottom
van Gieson × 184

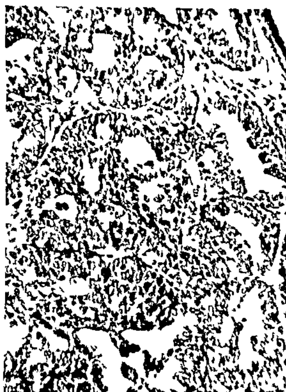


Fig 3 Nipple tumour Area with more florid cell proliferation larger, cylindrical cells with nuclear polymorphism and prominent nucleoli
Htx eos × 184

was found in either breast – The right axillary lymph nodes showed tumour growth of varying type Some parts had a dense hyaline stroma with nests of cells in partly cribriform pattern (Fig 4 A), others had papillary projections with large cylindrical cells and scanty stroma (Fig 4 B) Both types of metastases were like different parts of the nipple tumour

Para aortal lymph nodes contained areas of poorly differentiated tumour with nests of cells in marginal sinuses and invading the lymphatic tissue Vertebral metastases showed nests and strands of cells in hyaline stroma (Fig 5) The other tumour metastases were less well differentiated and grew like medullary carcinoma, in sheets of rounded cells with moderate polymorphism *e g* in the liver (Fig 6) Except for small nests of tumour cells in thin lymphatics in the lungs no other unexpected tumour was found at microscopy





Fig 4 A Axillary lymph node at necropsy Dense stroma and groups of glandular spaces with rather uniform cubical cylindrical epithelium
Htx eos × 75

Fig 4 B Axillary lymph node at necropsy Nests of tumour with papillary projections with scanty stroma and large, cylindrical cells with large nuclei
Htx eos × 115



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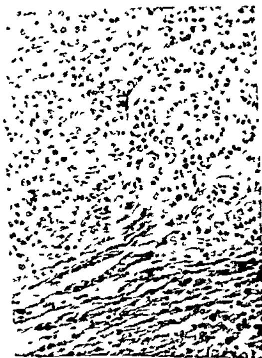


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Acta path microbiol scand Section A 79, 681–683, 1971

TRANSACTIONS OF THE SWEDISH PATHOLOGICAL SOCIETY

Meeting Borås April 17, 1971.

Gorel Östberg POLYARTERITIS NODOSA IN A CHILD

After a fortnight's illness with fever around 38° C and a diffuse rash a boy of nineteen months suddenly fell unconscious at home with respiratory and cardiac arrest Attempts at resuscitation during transport and for some hours in hospital were fruitless

Necropsy revealed signs of acute cardiac decompensation with pulmonary oedema, left sided hydrothorax and congestion of liver, spleen and kidneys The coronaries were greatly widened and had a tortuous appearance In the left descending branch there was a fresh thrombus about 1 cm in diameter There was an acute infarction in the anterior wall of the left chamber

Microscopy revealed inflammatory changes in the coronaries with widespread destructions in the vessel walls the elastic lamina had mostly disappeared and there was a granulation tissue with histiocytes some round cells and plymorphonuclear leucocytes Some smaller arteries in other organs showed similar arteritis

Polyarteritis nodosa occurs in all ages but is rather rare in children, a little more than 100 cases are described in detail It appears as an uncaracteristic illness with fever in a majority of cases rash and sometimes gastro intestinal or renal manifestations Patho-anatomical changes in the heart are more frequent than in adults The clinical diagnosis is often uncertain

Gertie Grossmann Bengt Robertson and Sigrid Soderlund LUNG BIOPSY DIAGNOSIS OF SECONDARY VASCULAR LESIONS IN CONGENITAL HEART DISEASE

Pulmonary vascular lesions, secondary to congenital heart disease can be diagnosed in biopsy material, usually obtained from the lingula or right middle lobe It has been claimed, however, that biopsy material from the lingula would give a false impression of medial hypertrophy in muscular pulmonary arteries, because of a normal over representation of muscularized arterioles in this lobe as compared with the rest of the lung (Heath & Best, J Path Bact 76 165, 1958) In order to test the validity of this statement, we compared the media index (media thickness/external diameter) of muscular pulmonary arteries and the relative number of muscularized arterioles (external diameter $<100\,\mu$) in various lung lobes in a consecutive autopsy series of 10 infants, varying in age between 1 day and 3 years There was a good correlation ($r = 0.89$, $p < 0{,}005$) between the values for media index in postmortem "biopsy" material from the lingula and the right middle lobe on one hand, and routine sections from the upper and lower lobes on the other The relative number of muscularized arterioles in the lingula did not deviate significantly from that in other lung lobes ($p > 0.1$) Biopsy material from the lingula lobe thus seems representative of the entire lung, at least in regard to the degree of medial hypertrophy in muscular pulmonary arteries

Ingvar Dahl, Lennart Angervall, Sonja Magnusson & Bertil Stener NODULAR FASCIITIS

During the last 5 years, at the Pathology Department, Gothenburg, Sweden, we have diagnosed 13 cases of nodular fasciitis, and to these, 12 cases have been added following retrospective examination of different skin lesions earlier diagnosed as *e g* fibrosarcomas, fibroxanthomas, xanthomas neurofibromas and malignant neurogenic tumours

The series consists of 12 females and 13 males ranging from 2 to 68 years of age The lesions were most often localized to the extremities, particularly to the forearm and the thigh. The clinical history revealed a rapidly growing tumour which in some cases was tender and painful The lesions were well

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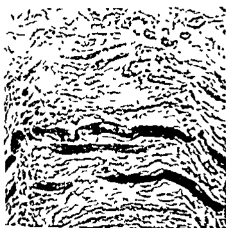


Fig 1 Longitudinal section from the vagus nerve of patient nr 1 Only a few myelinated fibres stain. Luxol fast blue-cresyl violet stain

with luxol fast blue and cresyl violet (13), haematoxylin van Gieson periodic acid Schiff (14) and with silver according to *Palmgren* (15)

Results and Comments

Marked lesions were present in the vagus nerves from all the three diabetic patients There was no essential difference between these cases with regard to the quality of the lesions The myelin sheaths were severly destroyed (Fig 1 and there was some crowding of Schwann cell nuclei in the same regions. The degeneration of myelin was partly of segmental character i e degenerated parts were surrounded by morphologically normal myelin in adjacent segments

Many axons appeared to be lost and several of the remaining ones were broken up into granular fragments. The amount of endoneurial connective tissue appeared to be increased The small vessels in and around the vagus nerve had thickened walls with varying degree of reduction in the size of the lumen As in other parts of the nervous system the vascular walls stained intensely red with the periodic acid Schiff procedure

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Summary

Patho-anatomical changes in the vagus nerve of three autopsy cases of diabetes mellitus with long duration of the disease are described

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BRIEF REPORTS

CHANGES IN THE VAGUS NERVE IN DIABETES MELLITUS

Krister Kristensson, Claes Nordborg, Yngve Olsson and *Patrick Sourander*

Various neurological disturbances may seriously complicate diabetes mellitus (1, 2, 3) Although the most common manifestations derive from the peripheral somatic nervous system symptoms and signs from cranial nerves and the autonomous nervous system may also arise during the course of the disease

Among the various autonomous dysfunctions in diabetes mellitus gastro-intestinal abnormalities are particularly common (1, 4, 5) The disturbed function of the gastro intestinal tract may produce symptoms of constipation and diarrhoea Roentgenologically, a delayed gastric emptying has been described (5) and the gastric secretion of acid may also be reduced (6) A loss of vagal stimulation has been discussed as an important pathogenetic factor for both phenomena

Previous studies on the pathology of the nervous system in diabetes mellitus have most often been restricted to peripheral somatic nerves (for ref see 3, 7) and rather few reports concern the morphology of other parts of the nervous system However, changes in the central (7, 8) and in the sympathetic nervous system (9, 10 11, 12) of diabetic patients have recently been described This report concerns lesions observed in the vagus nerve of three patients suffering from diabetes mellitus

Material and Methods

Case 1 (UB, 74/66) Female born 1935 with insulintreated diabetes since 1943 She had a severe diabetic retinitis and hypophysectomia was performed in 1958 Since 1962 her main symptoms were frequent diarrhoeas, sometimes with incontinence of the faeces No signs of malabsorbtion and no achlorhydria were found but an X ray of the ventricle showed signs of atonia She had also marked postural reactions The musculature was atrophic and in 1965 the achilles tendon reflexes were absent, two years later the patellar tendon reflexes were lost During her last year of life signs of renal insufficiency (serum—creatinine 1 7 mg per cent) and hypertonia (up to 200/115 mg Hg) developed She finally had a hypoglycaemic coma and died at an age of 31 years Autopsy revealed moderate atherosclerosis, diabetic nephropathy and necrosis of renal papillae

Case 2 (BH, 44/67) Male born 1927 with insulintreated diabetes since 1951 He was treated three times for hypoglycaemic coma and diabetic retinitis was observed in 1962 He had reduced sensibility of vibration in the toes and in his last year of life absence of achilles tendon reflexes and paresthesia in the legs During the last three years progressive renal insufficiency and hypertonia developed He also complained of increasing impotence Some episodes of nausea and vomitings were recorded and finally he had bleedings from ulcers in the ventricle and duodenum No other gastro intestinal symptoms were recorded He died in a state of uraemia in 1967 Autopsy showed a marked atherosclerosis of the coronary arteries and of aorta, cardiac hypertrophy, healed ulcers in the ventricle and diabetic nephropathy

Case 3 (BE 86/87) Male born 1929 with insulintreated diabetes since 1942 Diabetic retinitis and absence of achilles tendon reflexes were observed in 1962 No signs of renal failure or hypertonia were noted No gastro intestinal symptoms were recorded Since 1961 he had severe angina pectoris and signs of myocardial infarction developed in 1962 He died from a second infarction in 1967 Autopsy revealed advanced atherosclerosis of the coronary arteries with occlusion of the left circumflex branch multiple old and recent myocardial infarctions cardiac hyperthrophy aspiration and healed pyelonephritis

Histological Technique

Autopsy specimens were taken from the vagus nerve at a level corresponding to the middle part of the oesophagus Control specimens (autopsy) were also taken from patients without any history of diabetes mellitus

The specimens were fixed in 10 per cent formalin and embedded in paraffin Sections were stained

Received 6 viii 71 from *Neuropathological Laboratory, Institute of Pathology, University of Goteborg, Sweden*

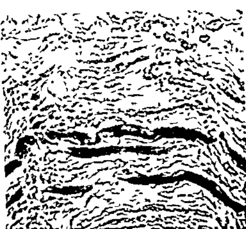


Fig 1 Longitudinal section from the vagus nerve of patient nr 1 Only a few myelinated fibres remain Luxol fast blue cresyl violet stain

with luxol-fast blue and cresyl violet (13), haematoxylin van Gieson, periodic acid Schiff (14) and with silver according to *Palmgren* (15)

Results and Comments

Marked lesions were present in the vagus nerves from all the three diabetic patients There was no essential difference between these cases with regard to the quality of the lesions The myelin sheaths were severly destroyed (Fig 1) and there was some crowding of Schwann cell nuclei in the same regions The degeneration of myelin was partly of segmental character *i e* degenerated parts were surrounded by morphologically normal myelin in adjacent segments

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THE EFFECT OF ZINC ON CALVES WITH HEREDITARY THYMUS HYPOPLASIA (LEHTAL TRAIT A 46)*

E Brummerstedt, T Flagstad A Basse and E Andresen

Recently a lethal trait was detected in Black Pied Danish Cattle of Friesian descent (1, 2) The syndrome develops in calves having a double dose of a lethal factor with autosomal recessive inheritance The symptoms begin when the calves are around 4–8 weeks old and the animals usually die within 4 months of age The main symptoms are exanthema with loss of hair on the legs and certain parts of the body and parakeratosis around mouth, eyes and under the jaw Several animals contract diarrhoea, conjunctivitis, rhinitis and bronchopneumonia At post mortem examination a hypoplastic thymus has been a characteristic and constant feature

It is an old observation that ZnO has a favourable effect on various skin diseases Several authors have reported on the effect of large doses of ZnO and $ZnCO_3$ on skin lesions associated with a syndrome similar to that observed in the Danish calves (3, 4, 5, 6, 7) It was therefore of interest to study the effect of zinc treatment on affected Danish calves with special reference to the possible influence on thymus

Four calves 1½ to 2½ months of age and exhibiting the typical lesions were selected for the experiment Two calves (467, 496) were given 0 5 gm ZnO orally daily until slaughtered five months after the start of the experiment, while two (465 466) served as untreated controls

After 7 to 10 days of treatment a significant effect was noted in the ZnO treated calves Their general state improved and the skin lesions were healing An ostensible complete recovery was seen after 1 to 2 months At the start of the experiment the weights of the calves were 56 and 39 kg Three months later the weights were 131 and 106 kg

In the two controls no improvement was seen There was little weight gain (49 to 66 kg and 45 t 70 kg in 3 months) and their general state an the skin lesions remained typical for the diseas Diarrhoea and bronchopneumonic manifestation were frequent Both calves died four months afte the start of the experiment

Post mortem examination of the two non treate calves (465, 466) showed lesions which have bee characteristic of all animals with the lethal trait i e, hypoplasia of thymus (25 gms resp 24 gms) and Peyer s patches and pronounced parakeratosi of the skin One of the zinc treated calves (496) showed morphologically normal thymus (377 gms) Peyer s patches and skin The other (467) had a thymus weight of 82 gms, normal Peyer s patches and mit ıl parakeratotic lesions

Since the calves increased in weight during the zinc therapy while the zinc dose was kept constant the amount of ZnO per kg body weight was ever decreasing This might explain the partial relapse seen in no 467

Although the experimental material is small the results give suggestive evidence for a repairing

are unable to utilize the amount of zinc ion normally available in the food and that this is important for a normal development of thymus

Since the results of thymus hypoplasia is dysfunction of the cell mediated immune response and certain parts of the humoral response further experiments to study the localization of the defect in zinc metabolism and the relationship between the zinc ion and the immune response are in progress as well as experiments in which ZnO is given in doses according to weight

If the syndrome in calves has a parallel among the hum
excluded
favourable e

Received 20 viii 71 *from Departments of Veterinary Virology and Immunology, Special Pathology and Therapeutics, General Pathology and Pathological Anatomy, and Animal Genetics, Royal Veterinary and Agricultural University, Copenhagen, Denmark*

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METHYLCHOLANTHRENE INDUCED MOUSE SKIN CARCINOGENESISS MODIFIED BY TREATMENT WITH POLYINOSINIC POLYCYTIDYLIC ACID (POLY I C)

Miklos Degré and *Kjell Elgjo*

Methylcholanthrene (MCA) is a complete carcinogen that has been extensively tested (16) Repeated topical applications to the mouse skin result in the development of papillomas and carcinomas and the mortality is high

Interferon inducers especially the double stranded RNA analogous polyinosinic polycytidylic acid (poly I C) appear to have an antitumorigenic effect Thus treatment with poly I C will protect the mice and inhibit the development of virus induced malignant tumours such as murine leukaemia and sarcoma (10 14 15) It has also a protective effect in mice inoculated with virus-containing tumour cells (7) Transplanted tumours are influenced by poly I C in some systems (1, 2, 12) but the compound has no effect in others (11) Recently tumour formation in two stage skin carcinogesis with dimethylbenz(a)anthracene as the initiating agent was found to be inhibited by poly I C (6) In this study we present some data on the influence of poly I C on mouse skin carcinogenesis induced by repeated topical applications of MCA

Three groups of hairless mice (hr/hr) 16–17 animals in each group were treated with an 0 5 per cent solution of MCA in benzene Five drops of this solution were applied twice weekly to the interscapular area Group I was treated with weekly intraperitoneal (i p) injections of 40 μg (0 1 ml) poly I C (Sigma Chemical Co) Group II was given an equivalent volume of saline until the appearace of the first tumours, when it was injected with poly I C once weekly Group III received 0 1 ml saline i p once weekly during the whole experimental period An additional group (IV) was not treated with MCA but injected with 0 1 ml poly I C once a week The mice were inspected at regular intervals The mortality was recorded daily After death, the tumours were removed fixed in formalin, and processed routinely Only tumours infiltrating the muscle layer (*panniculus musculosus*) were registered as carcinomas

The first tumours were observed 8 weeks after starting the MCA-*treatment* The tumorigenesis was slightly delayed in the animals that received poly I C but eventually tumours appeared in practically all animals treated with the carcinogen The mean induction time in Group I was 77 8 days in Group II 75 5 days, and in Group III 71 6 days No tumours were seen in Group IV that received only poly I C We found a somewhat lower incidence of tumours in the poly I C treated groups on the first two observations, *i e* 8 and 9 weeks after the first application of MCA Later the number of tumours per animal alive was not reduced by the poly I C treatment. The ratio between papillomas and carcinomas was not significantly altered by the poly I C treatment.

All MCA treated mice died within 135 days after the start of the experiment. Fig *1* shows, however, that the mortality was significantly

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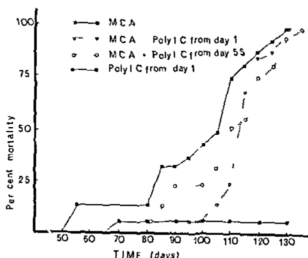


Fig 1 Accumulative mortality of mice treated with methylcholanthrene (0 5 per cent) twice weekly Poly I C was given once weekly (40μg) as indicated on the chart.

delayed in the poly I C treated Group I ($p<0\ 05$), and to a lesser degree in Group II The mean survival time in Group I was 113 5 days, in Group II 108 0 days, and in Group III only 97 0 days All mice but one survived in Group IV that received poly I C only These mice looked healthy and their weight kept within the normal range Random mice from Group IV were killed toward the end of the experimental period No pathological alterations were found at autopsy and histological examination of the spleen, the liver, the lungs, and the kidney revealed no signs of any toxic effect

Our findings indicate that repeated *i p* administration of poly I C delayed the tumorigenesis in mice, induced by repeated topical applications of MCA The effect was moderate but it should be emphasized that the MCA treatment was intensive and aimed at obtaining tumours in all animals within a short period of time A dramatic effect on the development of the tumours could hardly be expected under these circumstances

The mechanism of poly I C action can only be speculated upon at present Treatment with poly I C brings about a complex response in the organism It induces interferon production (5), increases the immunological capacity (3, 9), and influences RNA and protein metabolism both in normal and in neoplastic cells (13) Several factors of this response could be involved in suppression of tumorigenesis and it is difficult to get a clear picture of the present situation Thus, there seems to be no certain correlation between interferon production by poly I C and its antitumorigenic effect (18) Recent findings have indicated that poly I C induced interferon production in mice is reduced for several days after a single subcutaneous administration of MCA (17) In untreated hairless mice interferon was present from 4 hr to 5 days after a single *i p* injection of 40μg poly I C (for details about the method see ref 4) The relative importance of this interferon induction as compared to the other modes of host response after poly I C treatment remains to be clarified *Conclusion* After repeated topical applications of methylcholanthrene weekly administration of 40μg poly I C slightly delayed the development of tumours and increased significantly the survival time The poly I C treatment had no effect upon the final number of tumours, the ratio between carcinomas and papillomas, or upon the final mortality The weekly administration of poly I C had no apparent toxic effect on the mice

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